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MARRIAGE FERTILITY AND CANCER MORTALITY OF THE SPECIFICALLY FEMALE ORGANS: MAMMARY CARCINOMA.

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THE purpose of this study is to consider the possible relationship between marriage fertility and cancer mortality of the specifically female organs. In order to study this problem the marriage fertility in the various Netherlands provinces was compared with the average carcinoma mortality of the specifically female organs in those areas.

All mortality data were obtained from the Central Bureau of Statistics at the Hague.

The Netherlands consist of 11 provinces: Groningen, Friesland, Drenthe, Overijssel, Gelderland, Noord-Holland, Zuid-Holland, Utrecht, Noord-Brabant, Zeeland and Limburg.

As geographical units the provinces were selected for the following reasons:

(1) The Central Bureau of Statistics of the Netherlands publishes practically all their statistical data as provincial figures.

(2) Each province has a sufficiently large number of inhabitants to warrant statistical conclusions; this does not apply to each city or village separately. In our study only the cancer mortality figures were used because the cancer morbidity is not yet sufficiently known. This undoubtedly causes certain errors in our analysis. However, in the Netherlands with their good medical facilities this problem causes less difficulty for mammary carcinoma than for other sites of carcinoma or other diseases, because in a disease like mammary carcinoma practically no erroneous diagnoses are made.

Moreover our study represents only a comparative analysis and there is no reason to assume that the ratio between breast cancer mortality and morbidity is different in the various provinces.

The marriage fertility data also create certain problems. According to the definition of the C.B.S., marriage fertility is the number of children born per 1000 married females between the age of 15 and 49. The number of pregnancies ending in abortion are not considered in this case. As we are dealing with comparative studies the effect of this inaccuracy in our data will be small. Also the difference in abortion frequency for the various provinces does not affect the marriage fertility to such an extent that the succession of the provinces, if arranged according to fertility, would change, The more so, as we are dealing

with the marriage fertility of about 40 years ago, i.e. a period during which *abortus provocatus* was less frequent than in more recent years.

All our conclusions, based on the statistical data, were tested mathematically by Mr. H. Wijker of the Cancer Institute Anthoni van Leeuwenhoekhuis at Amsterdam.

In our studies the period 1946–1950 was considered, which has the advantage that in 1947 a population census was held. During the period 1946–1950, 6017 cases of mammary carcinoma mortality were reported in the Netherlands.

The mamary carcinoma mortality per year per 10,000 females (females married —or married in the past—and single respectively) for the various age groups is as follows :

TABLE I.—*Mammary Cancer Mortality per Year per* 10,000 *Females.*

	Age group.										
	30–34.	35–39.	40–44.	45–49.	50–54.	55–59.	60–64.	65–69.	70–74.	75–79.	80–84. 85, etc.
Single . .	0·24	1·38	2·49	3·89	8·15	11·14	11·16	16·87	18·57	20·48	38·97 32·85
Married . . (or married in the past)	0·48	1·29	2·37	4·35	5·39	6·69	8·55	10·19	12·94	13·90	16·78 21·14

Table I indicates the following :

(1) Mammary cancer mortality increases with age. In case of single females the increase seems to stop temporarily around the age of 60.

(2) Single women seem to have a greater chance of dying from mammary carcinoma than married ones. Below the age of 50 the number of deaths from mammary carcinoma in single women is very small, only 177 ; therefore the equal death rate between married and single women below the age of 50 has no statistical significance.

The author determined, for each province and for married and single women above the age of 30 separately, the number of deaths from mammary carcinoma to be expected, if in each province the average cancer mortality of the country as a whole (Table I) prevailed. These expected death-rates were compared with the actual observed death figures of each of the provinces (Table II).

TABLE II.—*Number of Mammary Carcinoma Deaths in the Various Provinces Observed and Expected.*

Province	Married.		Single.	
	Observed.	Expected.	Observed.	Expected.
Groningen . .	236	257	44	45
Friesland . .	269	266	57	46
Drenthe . .	106	128	18	16
Overijssel . .	283	315	55	55
Gelderland . .	441	508	97	121
Utrecht . . .	337	286	80	78
N.-Holland . .	1124	1008	207	204
Z.-Holland . .	1295	1232	297	270
Zeeland . . .	159	155	37	34
N.-Brabant . .	443	463	147	145
Limburg . .	220	272	50	76
	4913	4890	1089	1090

Table II warrants the following conclusions, which were confirmed by a mathematical statistical analysis :

(1) In the case of married women a clear relationship seems to exist between locality (province) and mammary carcinoma frequency.

(2) For single women the locality does not seem to affect the cancer frequency, except in the province of Limburg. The reason for this large discrepancy between observed and expected cancer frequency in the province of Limburg will be studied later on.

If we consider the various factors which are different for married and single women in the various provinces and which could explain the relationship between locality (province) and mammary cancer frequency in the case of married women, one is inclined to think of a factor related to the marital status of women, varying in intensity for different women. One of the most important factors seems to be the marriage fertility factor.

In order to study the possible relationship between marriage fertility and mammary carcinoma, for each province separately the mammary carcinoma frequency per 1000 married women was calculated for a period of 5 years and for each of the 5-year age groups separately.

The age groups were taken between 45 and 79 years for the following reasons :

(1) In the age groups below 45 and above 80 only a few mammary carcinoma cases occur in each province, which makes the figures unreliable from a statistical point of view.

(2) Also the marriage fertility is determined for a period of 35 years (15–49)

The result of our analysis is compiled in Table III.

TABLE III.—*Mammary Cancer Frequency per* 1000 *Married Women per Province for each* 5*-Year Age Group Separately.*

	Age group.						
	45–49.	50–54.	55–59.	60–64.	65–69	70–74.	75–79
Groningen	2·01	2·27	5·02	3·67	4·79	4·34	4·37
Friesland	1·43	2·04	2·98	4·66	5·79	8·72	7·24
Drenthe	2·63	2·83	3·72	3·19	3·54	3·94	4·72
Overijssel	2·16	3·11	2·68	3·41	2·18	7·08	7·25
Gelderland	1·93	2·99	3·78	4·62	4·05	4·49	5·42
Utrecht	2·67	2·57	4·01	4·25	6·92	8·98	7·72
N.-Holland	2·25	2·78	3·43	4·94	5·52	7·59	8·31
Z.-Holland	2·37	2·69	3·56	4·42	5·56	6·50	7·98
Zeeland	2·34	2·77	2·93	3·34	6·73	6·67	7·99
N.-Brabant	2·03	2·91	2·38	4·16	4·74	6·24	5·31
Limburg	1·83	2·12	3·23	2·89	4·06	4·77	5·08
Country (as a whole)	2·17	2·69	3·34	4·27	5·09	6·46	6·95

Using a standard population for each province and the country as a whole, i.e. 1000 married women in each age group between 45 and 80, the total number of mammary carcinoma cases per province can be found in Table III by simply adding the various age group figures, for each province separately. If these provincial standardized figures are arranged according to increasing figures the following provincial succession is found as indicated in Table IV :

It is evident that the recent mammary carcinoma cases in married women should not be correlated with the present day marriage fertility in the various provinces but with a period about 30 years ago, when the female population referred to in Table IV varied in age between 15 and 50 years. It is this age group which determines the marriage fertility.

In *First Results of an Analysis of the Birth-rates in the Netherlands* (Publication

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TABLE IV.—*Mammary Carcinoma Frequency in the Various Provinces and the Country as a Whole for a Standardized Female (Married) Population.*

Limburg	23·98
Drenthe	24·57
Groningen	26·47
Gelderland	27·28
N.-Brabant	27·77
Overijssel	27·87
Country (as a whole)	30·97
Zeeland	32·77
Friesland	32·86
Z.-Holland	33·08
N.-Holland	34·82
Utrecht	37·12

No. 2 of the Institute for Social Research in the Netherlands, Amsterdam, December 1949) Drs. v .d Brink publishes a curve indicating the marriage fertility for the various provinces and the country as a whole for the period 1870–1940. From this curve the following figures can be induced (Table V).

TABLE V.—*Marriage Fertility in* 1917.

N.-Brabant	280
Limburg	272
Drenthe	250
Gelderland	220
Overijssel	205
Utrecht	203
Country as a whole	202
Groningen	190
Z.-Holland	190
Zeeland	185
Friesland	182
N.-Holland	165

A comparison between Tables IV and V suggests a correlation between marriage fertility and mammary carcinoma of married women, which could be confirmed by a mathematical statistical analysis of the data of Tables IV and V.

The discrepancy between marriage fertility and mammary carcinoma in the province of Utrecht, may be explained by the fact that this province has a much higher percentage of recently settled population (especially older people) arrived from other provinces, than any of the other provinces.

The observed inverse relationship between marriage fertility and mammary carcinoma can also be demonstrated in a different way :

(1) Roman Catholic married women represent 38·5 per cent of the total married female population. However only 32·7 per cent of all mammary carcinoma cases are found in this Catholic group. In the Dutch Reformed married women, representing 31·5 per cent of the total married female population. 41·2 per cent of all mammary carcinoma cases are found. In the Roman Catholic population the birth frequency is above the average of the country, in the case of the Dutch Reformed it is below the average.

(2) If all the Netherlands municipalities with a high birth frequency (36 per mille or more) are taken together, one finds 31 cases of mammary carcinoma in 67,115 inhabitants. If we do the same for municipalities with a very low birth frequency (17·4 per mille and less) 74 cases of mammary carcinoma are recorded in 67,985 inhabitants. According to the average population in the Netherlands, in

67,000 inhabitants only 50 cases of mammary carcinoma are expected. In other words, here again we notice an inverse relationship between marriage fertility and mammary carcinoma of married women.

All these facts indicate a pronounced inverse correlation between marriage fertility (resp. birth frequency) and mammary carcinoma (marriage fertility and birth frequency running parallel). As mammary carcinoma is more frequent amongst single women, we are inclined to assume that pregnancy, or the lactation factor related to it (O. Mühlbock, 4th Dutch Yearbook of Cancer 1954, page 47), has a certain preventive action on the development of mammary carcinoma.

The observation that for a group as a whole high marriage fertility correlates with low mammary carcinoma frequency does not permit the conclusion that this applies to each individual separately and that for a married woman the chances of developing mammary carcinoma would decrease with an increasing number of children ; for the marriage fertility indicates the number of children born from all married women between the ages of 15 and 49. This includes also the married women without children and therefore the marriage fertility figure depends as well on the number of childless marriages as on the number of children per family with children.

Still it seems true that for each individual married woman a period of pregnancy, followed by lactation, creates a preventive action against mammary carcinoma. However, whether this preventive action in the individual person increases with increasing number of pregnancies requires further study.

The problem arises whether the observed inverse correlation between marriage fertility and mammary carcinoma frequency could be expressed in figures.

If the marriage fertility in the total population were equal to the three most fertile provinces, N.-Brabant, Limburg and Drenthe, and considering the data of Table III one must assume that, in the Netherlands, between the ages of 45 and 79, 3434 cases of mammary carcinoma would occur.

If we do the same on the basis of the province with lowest marriage fertility (i.e. N.-Holland) the expected number of mammary carcinoma cases would be 4406. In other words, a decrease in marriage fertility by 100 increases the mammary carcinoma frequency by 1000. The observed marriage fertility of the country, equal to 202, would require 4064 cases of mammary carcinoma. In reality this figure amounts to 3988.

If the marriage fertility should be 0, as in the case of married women without children, we could expect $(4406 + \frac{165-0}{100} \times 1000)$ cases of mammary carcinoma amongst married females between 45 and 79. This carcinoma frequency approaches closely that for single women, but not completely because there is always a small percentage of single women with one or more children. If we calculate, on the basis of Table I, the number of cases of mammary carcinoma which could be expected if the total female population were unmarried, we find the figure 5702, which is close to the above-mentioned figure of 6056.

On the other hand, with a marriage fertility of 256, 3434 cases of mammary carcinoma are observed. This figure would be reduced to zero if the marriage fertility were increased by 340, in other words, to a fertility of about 600. With such a high fertility (600), which would mean that each married woman during her fertile period would produce 3 children every 5 years, no mammary carcinoma should occur. (Fig. 1.)

It seems therefore that the observed correlation between marriage fertility and mammary carcinoma mortality can be expressed in exact figures. However, whether the observed decrease of marriage fertility by 100, concurrently with a mammary carcinoma frequency (of married women) increased by 1000, can be considered to be a general rule or is only applicable to the Netherlands and its provinces will be an object of further mathematical study.

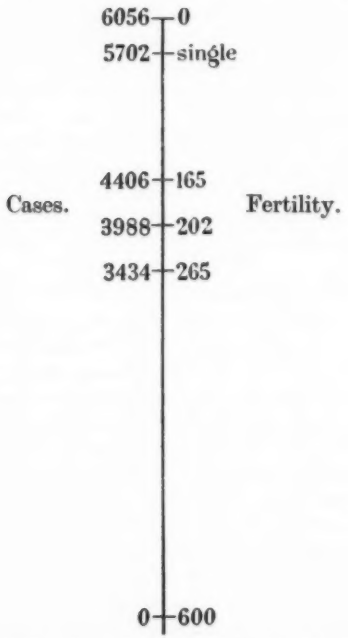

FIG. 1.—Relationship between marriage fertility and mammary carcinoma of married women between the ages of 45 and 79.

The observed correlation between marriage fertility and mammary carcinoma mortality warrants the following practical conclusions :

(1) During the last decades marriage fertility in the Netherlands has decreased considerably. If all other factors remain the same we must expect that in married women the mammary carcinoma mortality would increase considerably. A comparison between the mammary carcinoma mortality figures of the periods 1931–1935 and 1946–1950 seems to confirm this expectation. The number of deaths from mammary carcinoma of married women increased by 16·3 per cent. However, the same applies to the unmarried women, the increase being 14·7 per cent. The increase is different for the various age groups. If, for the various age groups, the death-rates from mammary carcinoma during the period 1946–1950 are expressed as percentages of the corresponding death-rates for the period 1931–1935 the figures given in Table VI are obtained.

The decrease in the youngest age group (40–49) seems to be less pronounced in the case of married women (perhaps a therapeutic effect?) ; however, the increase

TABLE VI.—*Mammary Carcinoma Death-rates* 1946–1950 *as Percentages of Corresponding Death-rates* 1931–1935.

	Age Groups.			
	40–49.	50–59.	60–69.	70–79.
Single women :				
Period 1931–1935 . .	100	100	100	100
„ 1946–1950 . .	77·1	97·9	108·9	143·4
Married women :				
Period 1931–1935 . .	100	100	100	100
„ 1946–1950 . .	90·2	91·1	113·8	147·3

in the two oldest age groups (60–69 and 70–79) is more pronounced in the married group. These figures suggest the influence of the decrease in marriage fertility on the increase in mammary carcinoma of married women. However this effect is hidden because the other factors, which determine the mammary carcinoma frequency, differ in both periods as demonstrated by the carcinoma figures for single women.

(2) For comparative geographical pathological studies in the different countries it will be necessary, in the case of mammary carcinoma, to consider seriously the differences in marriage fertility and birth frequencies of those countries.

This forces us to :

(*a*) Determine the mammary carcinoma frequency for married and single women separately. From an aetiological point of view, real differences between countries can be accepted only if the differences in mammary carcinoma can also be observed in single women.

(*b*) Take into consideration the preventive influence of high birth frequency or marriage fertility on the mammary carcinoma mortality of married women in each country.

(3) In genetic studies mammary carcinoma frequency of mothers or sisters of carcinoma patients is often compared with the average female population. However, as mammary carcinoma patients often belong to a female population group with low marriage fertility and, for social, religious or hereditary reasons, the patients usually resemble more closely the mother and sisters in their marriage fertility than the average female population of a country, one may expect a higher mammary carcinoma frequency amongst mothers and sisters of mammary carcinoma patients than in the average female population. This would hold even without the influence of a hereditary factor. In other words, a high mammary carcinoma frequency amongst relatives in itself is no indication of the possible hereditary nature of mammary carcinoma.

SUMMARY.

A study of the mammary carcinoma mortality indicates that in the case of single women the mortality figures for the various provinces do not differ, except in one province ; for married women an inverse correlation exists between marriage fertility and mammary carcinoma mortality, which shows up as a low mammary carcinoma mortality in provinces with high marriage fertility and *vice versa*. An accurate comparison of the mammary carcinoma mortality in different years in the same country and between different countries can be made only if the marriage fertility (or the birth frequency, being parallel with the fertility) is taken into consideration.

THE SEX CHROMATIN IN BENIGN TUMOURS AND RELATED CONDITIONS IN MAN.

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THE authors have shown in an earlier paper (Moore and Barr, 1954) that intermitotic nuclei of various human tissues have a distinctive morphology according to sex. The nuclei of *females* contain a mass of sex chromatin that is characteristically plano-convex in shape and adjacent to the inner surface of the nuclear membrane. It is about 1 μ in diameter. In sections 5 μ thick and stained with haematoxylin and eosin or by the Feulgen method, the sex chromatin is seen in about three-fourths of the nuclei. The nuclei of *males* seldom contain a mass of chromatin that is comparable to the sex chromatin of females in size, shape and position.

Since the sex chromatin is Feulgen-positive, indicating the presence of DNA, it is probably a chromosomal derivative. The size relationship to sex points to an origin from the sex chromosomes. It is thought that the X-chromosomes of female somatic cells have especially large heterochromatic regions, i.e. regions that remain compact and densely staining in the intermitotic nucleus, and that these portions of the two X-chromosomes adhere to each other to form the sex chromatin of female cells. If this interpretation is correct, it follows that the XY-chromosomes of male nuclei do not form a mass of chromatin of comparable size. The small size of the Y-chromosome is probably responsible, in part, for the inconspicuous nature of the sex chromatin in male intermitotic nuclei.

The sex difference in resting nuclei has been confirmed for human epidermis by Emery and McMillan (1954), Hunter, Lennox and Pearson (1954) and Marberger and Nelson (1954). The special interest in epidermal nuclei arises from the use of skin biopsies for the detection of chromosomal sex in congenital errors of sex development (Moore, Graham and Barr, 1953; Barr, 1954; Polani, Hunter and Lennox, 1954; Wilkins, Grumbach and Van Wyk, 1954; Sohval, Gaines and Gabrilove, 1955).

The apparent derivation of the sex chromatin from "heterochromatin" has prompted its study in tumour cells, since Caspersson and Santesson (1942) regard disturbances in the "heterochromatin" as a pre-requisite for malignant growth. This view is linked with the broader hypothesis, elaborated by Caspersson (1950) and others, that the nucleolus and "heterochromatin" are involved in some fundamental way in the synthesis of cytoplasmic proteins. The work reported here on benign tumours and similar conditions is intermediate between the study of normal tissues (Moore and Barr, 1954) and the study of malignant tumours, which is now in progress. A preliminary report of the observations made during the present study has appeared (Moore, 1955).

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MATERIALS AND METHODS.

Specimens of benign tumours, and of hypertrophic and hyperplastic tissues, were collected from surgical material principally. The ages of the subjects varied from 3 months to 81 years, but almost all of the specimens were from adults. There were 126 specimens, representing over 40 pathological diagnoses, from which satisfactory sections for the study of nuclear detail were obtained ; 65 specimens were from females and 61 from males.

A preliminary study of sections that had been prepared for routine pathological diagnosis showed that such preparations were often unsatisfactory for the study of fine nuclear detail. The particles of nuclear chromatin must be sharply defined. Sections are unsuitable for this work if there is any appreciable shrinkage and pyknosis of the nuclei or if the nuclei have a " ground glass " texture with little structural detail visible, other than the presence of nucleoli. Sections from fresh material were prepared, therefore, according to the following procedure.

Small blocks of tissue were fixed for 24 hours in the following solution : formalin 20 per cent, 95 per cent alcohol 35 per cent, glacial acetic acid 10 per cent, distilled water 35 per cent. After immersion in 70 per cent alcohol for 1–3 days, the blocks were dehydrated, cleared, embedded in paraffin, and sectioned at 5 μ. Sections from each specimen were stained with Harris's haematoxylin and eosin (Gatenby and Beams, 1950), and by the Feulgen method (Gurr, 1953) with a pale counter-stain of light green. Feulgen preparations are especially useful for study of the sex chromatin. Sections from some specimens were of poor technical quality, even when especially prepared, and had to be discarded.

In each specimen of satisfactory technical quality, 100 nuclei in sections stained with haematoxylin and eosin and 100 nuclei in Feulgen preparations were examined with an oil immersion objective for the presence of sex chromatin. Attention was directed to the type of cell that was primarily involved in the neoplastic growth. In order to obtain data on the relative size of the female sex chromatin in cells of benign tumours and in normal cells, two diameters of the sex chromatin at right angles to each other were measured with a filar micrometer eye-piece in a sample of 30 cells in each of 15 pathological specimens and 20 normal tissues.

OBSERVATIONS.

(a) *Nuclei of benign tumours and related conditions in females.*

The incidence of nuclei containing a mass of sex chromatin is shown in Table I. In some instances several specimens with the same diagnosis from different patients were studied ; here an average figure is recorded, with the number of specimens appearing in brackets. In the 65 benign tumours and similar conditions in females the sex chromatin was seen in from 66 to 82 per cent of nuclei, with an average incidence of 75 per cent. The average incidence of the sex chromatin in 18 normal tissues of female subjects was 74 per cent (Moore and Barr, 1954). Differences in the technical quality of the sections were probably responsible for most of the variation from one specimen to another.

The sex chromatin usually took the form of a plano-convex mass, adherent to the inner surface of the nuclear membrane. Nuclei in a cutaneous papilloma, a uterine leiomyoma, a thyroid adenoma and in a nerve cell of an ovarian dermoid cyst are illustrated in Fig. 1, 3, 4 and 5. The sex chromatin of smooth muscle

TABLE I.—*Incidence (per cent) of Sex Chromatin.*

Histogenic classification.	Pathological diagnosis.	♀.	♂.
Glandular epithelium	Gastric polypus	78 (2)	—
	Multiple polyposis of colon	78	11 (2)
	Rectal polypus	68	6 (6)
	Papilloma of urinary bladder	—	6
	Hypertrophy of prostate	—	6 (3)
	Endocervical polyp	66	—
	Fibroadenoma of breast	71 (2)	—
	Duct papilloma of breast	74	—
	Cystic lobular hyperplasia of breast	72	—
	Mazoplasia of breast	70	4 (2)
	Adenoma of sweat gland	75 (2)	9
	Sebaceous cyst	—	5 (3)
	Arrhenoblastoma	80	—
	Leydig cell tumour	—	4
	Chromophobe adenoma of pituitary	76	—
	Colloid adenoma of thyroid	69	4
	Foetal adenoma of thyroid	76 (2)	—
	Adenomatous goitre	77 (3)	15
	Parathyroid adenoma	—	18
Non-glandular epithelium	Cutaneous papilloma	75 (7)	6 (12)
	Senile keratosis	69 (2)	6
	Molluscum contagiosum	—	6
	Leukoplakia of buccal mucosa	73 (2)	4 (3)
	Papilloma of tonsillar fossa	—	9
	Condyloma acuminatum	73 (2)	—
Melanin-forming tissue	Intradermal naevus	79 (5)	7 (7)
	Compound naevus	75 (2)	—
	Blue naevus	77	—
	Junctional naevus	69	—
Vascular tissue	Capillary haemangioma	76	7
	Cavernous haemangioma	—	3
Muscle	Leiomyoma of uterus	79 (7)	—
	Leiomyoma of cervix	75	—
	Leiomyoma of broad ligament	79	—
	Leiomyoma of stomach	75	4 (3)
Connective tissue	Keloid	76	—
	Dermatofibroma	68	4 (3)
	Pedunculated fibroma of hard palate	71	—
	Chondroma	—	3
	Osteochondroma	67	4
Embryonal and mixed tissues	Dermoid cyst of forehead	—	2
	Branchial cleft cyst	71	4
	Mixed tumour of parotid	75 (3)	8 (2)
	Dermoid cyst of ovary	74 (4)	—

tumours was not located at the pole of the nucleus as often as it is in normal smooth muscle cells. With this minor difference, its position in the nucleus was similar in normal tissues and in the tumours.

Measurements of the female sex chromatin in normal cells of various tissues are recorded in Table II. The mean size, considering the tissues together, was 0·7 × 1·2 μ. The size of the female sex chromatin appears to be similar in monkey and man since Prince, Graham and Barr (1955) found the mean size of the sex chromatin to be 0·8 × 1·1 μ in various tissues of *Macacus rhesus*. Some of the size variation, as recorded in Table II, is undoubtedly caused by sampling and by errors in measuring such a small object. Nevertheless, we suspect that the female sex

chromatin is a little larger in some tissues than in others since it was found to be relatively large in the adrenal cortex, for example, in man, monkey and in the cat (Graham and Barr, 1952).

TABLE II.—*Size of the Sex Chromatin in Normal Tissues of Females.*

Tissue.	Mean size of sex chromatin (μ).
Epidermis	0·7×1·2
Umbilical cord (surface epithelium)	0·8×1·1
Surface epithelium, gastric mucosa	0·7×1·3
Surface epithelium, duodenal mucosa	0·6×1·1
Parenchymal cells of liver	0·7×1·3
Acinar and duct epithelium, pancreas	0·7×1·2
Convoluted tubules of kidney	0·6×1·2
Epithelium, urinary bladder	0·9×1·1
Stromal cells of ovary	0·8×1·2
Glandular epithelium of uterus	0·7×1·1
Large lymphocytes of splenic nodules	0·6×1·1
Cartilage cells	0·8×1·4
Skeletal muscle	0·7×1·3
Cardiac muscle	0·6×1·2
Smooth muscle of uterus	0·7×1·1
Pars distalis of pituitary	0·8×1·3
Adrenal cortex	0·9×1·3
Adrenal medulla	0·7×1·1
Thyroid epithelium	0·7×1·4
Islets of Langerhans	0·7×1·3
Average	0·7×1·2

Measurements of the female sex chromatin in cells of benign tumours and related conditions are shown in Table III. Considering the various abnormal tissues together, the average size of the sex chromatin was 0·8 × 1·2 μ. Such measurements on a considerable number of benign tumours and their tissues of origin would be necessary to learn whether the size of the sex chromatin is altered significantly in benign tumours. From the work done thus far it may be inferred that any possible difference must be very small and difficult to demonstrate.

TABLE III.—*Size of the Sex Chromatin in Benign Tumours and Related Conditions in Females.*

Tissue.	Mean size of sex chromatin (μ).
Cutaneous papilloma	0·8×1·2
Intradermal naevus	0·6×1·1
Junctional naevus	0·8×1·1
Senile keratosis	0·9×1·2
Keloid	0·9×1·1
Leukoplakia of buccal mucosa	0·8×1·2
Gastric polypus	0·8×1·3
Multiple polyposis of colon	0·8×1·2
Osteochondroma	0·8×1·4
Leiomyoma of uterus	0·7×1·3
Dermoid cyst of ovary	0·8×1·2
Mixed tumour of parotid	0·8×1·3
Chromophobe adenoma of pituitary	0·7×1·2
Adenomatous goitre	0·8×1·3
Foetal adenoma of thyroid	0·7×1·3
Average	0·8×1·2

(b) *Nuclei of benign tumours and related conditions in males.*

With one exception, noted below, the specimens obtained from male subjects showed a typical male nuclear structure (Fig. 2). A mass of chromatin that was rather larger than the other chromatin particles was seen in an average of 6 per cent of nuclei, varying in individual specimens from 2 to 18 per cent. One cannot be certain that these masses of chromatin represent the male sex chromatin derived from the XY-chromosomes. Although the figures for the incidence of sex chromatin in specimens from males are, on this account, only approximations they serve to illustrate the definite difference in nuclear structure between specimens obtained from females and those obtained from males. The figures are similar to those that we have recorded previously for normal male tissues, where a chromatin mass that might represent the sex chromatin of male nuclei was seen in an average of 8 per cent of nuclei, with the incidence varying in individual specimens from 1 to 21 per cent. In both normal tissues and benign lesions there is a wide range, between a high figure of about 20 per cent for males and a low figure of about 60 per cent for females, separating the incidence of sex chromatin in the two sexes.

A teratoma of the testis was studied but was omitted from Table I because it does not fall under the heading of benign tumours. However, the observations on this tumour are sufficiently significant to justify a brief reference to it at this time. This teratoma developed in the right testis of a 23-year-old and was removed at operation. It contained both epithelial and mesodermal elements. Much of the tumour was unsuitable for detailed cytological study. Certain areas were good for cytological detail, however, and in these favourable locations the nuclei had a typical female structure with a mass of sex chromatin occurring in about 70 per cent of the nuclei. The nucleus of a fibroblast-type cell in the teratoma, with typical female sex chromatin, is illustrated in Fig. 6.

DISCUSSION.

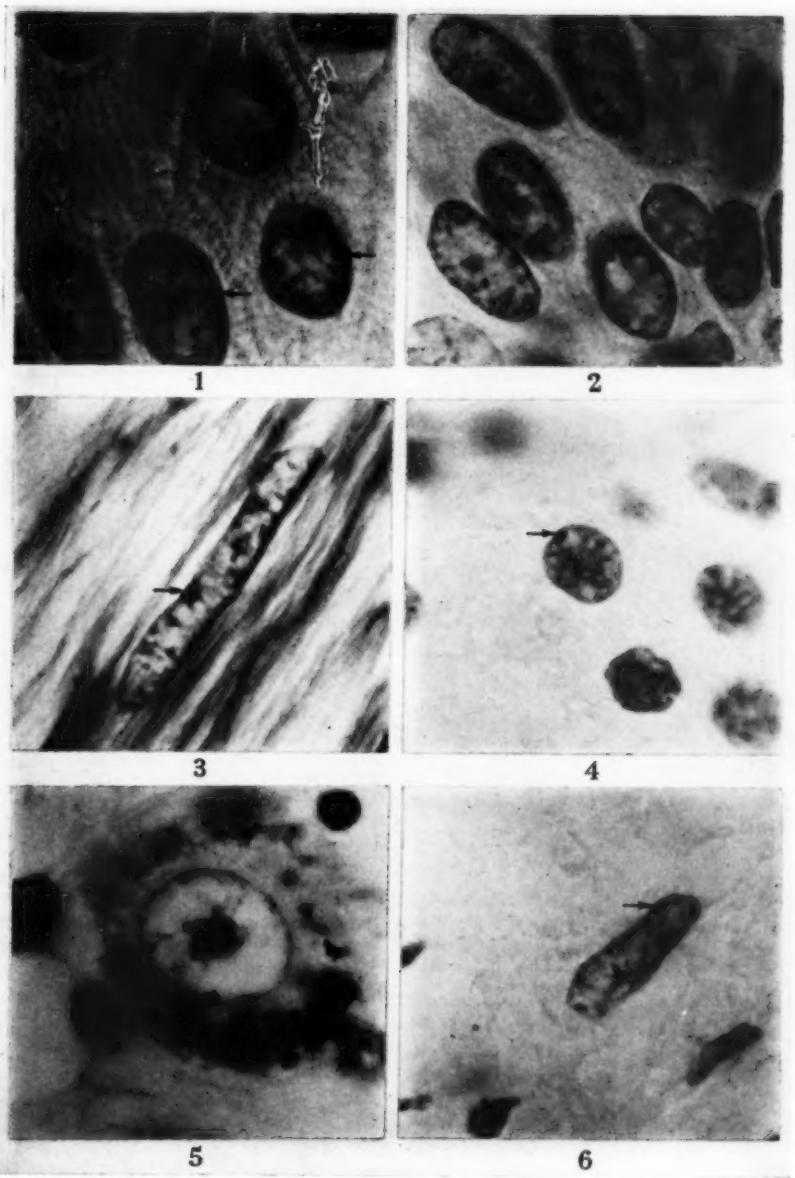
The nuclei of benign neoplasms are generally thought to be similar to those of normal tissues. They have been described as larger, in some instances, than nuclei of the normal tissue in which the neoplasm arose (Ehrich, 1936). However, the mitotic and chromosomal aberrations that may occur in malignant tumours have not been described as a feature of benign neoplasms. To this may now be added the further item that the nuclei of benign tumours and normal tissues in man are similar with respect to their morphological characteristic of sex.

The finding of typical female nuclei in an arrhenoblastoma of the ovary (Table I), recalls a histogenetic hypothesis which was advanced by Schiller (1940) for these tumours. He suggested that a cell may lose an X-chromosome during a mitotic division, thus acquiring the potentiality of producing a tissue along male

EXPLANATION OF PLATE.

(The magnification of all photomicrographs is × 2000. The sex chromatin is indicated by an arrow.)

FIG. 1.—Cutaneous papilloma, female. Feulgen stain.
FIG. 2.—Cutaneous papilloma, male. Feulgen stain.
FIG. 3.—Leiomyoma of uterus. Haematoxylin and eosin stain.
FIG. 4.—Thyroid adenoma, female. Feulgen stain.
FIG. 5.—Nerve cell in dermoid cyst of ovary. Cresyl violet stain.
FIG. 6.—Fibroblast in a teratoma of the testis. Feulgen stain.



Moore and Barr.

structural lines. The arrhenoblastoma in this series was submitted for study by Dr. A. R. Sohval, of New York City. It was a trabeculated type of arrhenoblastoma containing pseudotubular structures, and of excellent technical quality The interstitial cells had a typical female structure. It is inferred that they bear the XX-sex chromosome complex and that the hypothesis of Schiller does not apply to this particular specimen. A more detailed description of this tumour, together with an account of nuclear structure in a number of pathological conditions, will be published shortly (Sohval and Gaines, 1955).

The finding of female nuclei in a teratoma of the testis raises questions concerning the histogenesis of these tumours that cannot be answered at this time. Hunter and Lennox (1954) found that of 12 teratomata in women all had female-type nuclei, while of 9 teratomata in males 5 had female-type nuclei and 4 had male-type nuclei. Cruickshank (1955) studied squamous-cell formations in 5 mediastinal teratomata. Two of the hosts were female and the tumour nuclei were also female. Three of the hosts were male, and the tumour nuclei were male in two instances and female in the other. Because of these observations the view, currently discounted, that a teratoma may be a unique tumour arising from the fusion of gametes or other haploid cells merits renewed consideration.

SUMMARY.

Nuclei of benign tumours and related conditions in man were studied with respect to their sex characteristics. Sixty-five specimens were from female subjects and all showed typical female-type nuclei. Sixty-one specimens from males contained typical male-type nuclei. It is concluded that the sex characteristics of nuclei, i.e. the presence of a well-developed mass of sex chromatin in females only, is similar in benign tumours and related conditions and in normal tissues.

The finding of typical female-type nuclei in an arrhenoblastoma of the ovary discounts the hypothesis that these tumours are generally derived from a cell which has lost an X-chromosome. On the other hand, the presence of female-type nuclei in a teratoma of the testis re-opens the problem of the histogenesis of this type of tumour.

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THE LATE EFFECTS OF THOROTRAST ADMINISTRATION. A REVIEW AND AN EXPERIMENTAL STUDY.

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A STUDY of the late effects of intravenously administered thorotrast is one which is of considerable practical importance in view of the past use of this material as a contrast agent in radio-diagnosis, as well as being of great interest in relation to the carcinogenic action of radio-active particulate matter.

Thorotrast is a colloidal solution of thorium dioxide, containing 19–20 per cent of this compound, stabilized by Dextrine (15–20 per cent). The preparation contains methyl-p-hydroxybenzoate (0·15 per cent) as a sterilization agent. In the course of the disintegration of thorium and its daughter elements, α, β and γ radiations are emitted. When injected intravenously, the particles are taken up by the cells of the reticuloendothelial system and, as one would expect, the organs which show the greatest amount of thorotrast after injection are the liver, spleen, bone marrow and lymph nodes. Thorotrast is also found, sometimes in appreciable amounts, in the adrenal glands, kidneys and lungs.

According to McMahon, Murphy and Bates (1947), thorium dioxide was introduced in roentgen diagnosis by Frik and Bluhbaum (1928) who made the first experiments with the substance. Some time later Radt suggested its use in the visualisation of the spleen and liver and developed a suitable solution to be employed in human beings. With the work of Moniz (1934) and his collaborators on the clinical use of thorotrast in cerebral angiography the product became widely adopted.

Thorotrast seemed to be harmless as regards immediate effects, and appeared to be inert as far as biochemical processes were concerned. However, in 1932 the first case of damage (thrombosis) which could be ascribed to thorotrast was reported (Silva Horta, 1953), and in 1947 McMahon, Murphy and Bates published a report of what is thought to be the first case of malignancy related to thorotrast.

The present paper is divided into two parts. The first part is a critical review of the literature on the subject. The second part is the report of an investigation of the effect of intravenously injected thorotrast in the mouse.

PART I.

Review of the Literature.

From the work of Martland (1931) on the cases of osteogenic sarcoma occurring in Radium dial painters, and the subsequent work of many investigators using artificially produced radio-active materials (Furth and Lorenz, 1954) there is no doubt that a radio-active substance present in the body for a certain length of time may be the source of malignant changes in the tissue.

The introduction of thorotrast in radiological diagnosis aroused suspicions about its possible carcinogenic properties, but most clinicians were sceptical on account of its low activity and the small doses employed. Furthermore, short-term experiments carried out with animals (Tripoli and Haam, 1932; Irwin, 1932) seemed to support the belief that thorotrast was harmless under the conditions and in the doses then used.

Oberling and Guerin (1933) injected 1 c.c. of thorotrast intraperitoneally, twice a week for 5 weeks, into rats grafted with Jensen sarcoma, and found that in a number of animals the tumours regressed. Among the rats which had absorbed the tumours, 8 showed peritoneal sarcomatosis and the remaining 2 showed early sarcomatous proliferation. The histology was a little different from that of Jensen sarcoma and attempts to transplant were negative, in spite of the fact that this strain usually yields 100 per cent of takes of Jensen sarcoma.

Pohle and Ritchie (1934) criticised some previous papers in which thorotrast was considered to be harmless, mainly on account of the rather short period of observation of the animals used in the experiments. They carried out an experiment on rabbits in which the animals were observed and studied histologically from 2 to 493 days after the injection. The preparation was injected into the ear vein in doses varying from 0·25 to 0·5 to 1 c.c. per kilogram of body weight. The early changes in the liver were a hydropic degeneration mainly about the central vein and some oedema of the portal spaces. The late findings were scattered areas of atrophy of the parenchyma and slight, but definite, overgrowth of the connective tissue. In the spleen they found fibrosis of the pulp in the majority of the animals. The bone-marrow showed a picture of exhaustion developing two months after the injection. No lesions were found in the lungs, kidneys or adrenals. They did not find any decrease in the amount of thorotrast over the period of observation.

In the same year Roussy, Oberling and Guerin (1934) obtained 8 sarcomas in 15 animals which died between 15 and 17 months after subcutaneous and intraperitoneal injections of thorotrast. The animals received five injections of 1 c.c. given at intervals of 3 and 4 days. Two years later Roussy, Oberling and Guerin (1936) repeated the experiments using doses of 0·5 c.c. and confirmed their previous findings.

Selbie (1938) undertook an experiment using 60 rats and 60 mice, which were injected subcutaneously with two doses of 0·3 c.c. and two doses of 0·1 c.c. respectively. Among the rats, the tumours began to appear at 52 weeks and 25 tumours were found in rats which lived long enough to have them. Twenty-two were spindle cell sarcomas and the other 3 fibromas. Tumours appeared in 58 per cent of the rats. In the mice, the first tumour appeared at 39 weeks, and 9 tumours were obtained: 6 spindle cell sarcomas, an osteosarcoma, a histiocytoma and a capillary angio-endothelioma. Tumours appeared in 43 per cent of the mice. Selbie (1938) suggested that the carcinogenic activity of the thorotrast was probably due not only to its radio-activity but also to the susceptibility of the inflammatory tissue which it produces.

Orr, Popoff, Rosedale and Stephenson (1938) after studying the action of thorotrast injected intravenously in rabbits, concluded that the drug should not be used in human beings because of (1) its lack of elimination from the body, (2) its blockade of the reticulo-endothelial system which may thus adversely affect immunity mechanism, (3) the likelihood that it may profoundly damage the liver

TABLE I.—*Malignancy Related to Thorotrast.* (*Observations in Humans*).

Author.	Year.	Method.	Dose (c.c.).	Years after.	Type of tumour.	Comments.
McMahon, Murphy and Bates	1947	Hepatolienography	75	12	Endothelial cell sarcoma from liver	Syphilitic back-ground (gumma).
Zollinger	1949	Retrograde pyelography	30	16	Spindle cell sarcoma from kidney	Hydronephrosis and chronic pyelonephritis as primary diseases.
Rudolphi	1950	Dakryocystography	?	35	Squamous carcinoma from eyelid	Chronic inflammation present.
Abrahamson, O'Connor and Abrahamson	1950	Hepatolienography	75	16	Alveolar cell tumour from lung	No thorotrast was detected in the lungs.
Silva Horta	1953	Cerebral angiography	20	3 yrs., 2 mo.	Endothelial cell sarcoma from liver	No comments.
Matthes	1954	Hepatolienography	75	21	Liver cell carcinoma	No comments.

TABLE II.—*Malignancy Related to Thorotrast.* (*Observations in Animals.*)

Author.	Animal.	Route of injection.	Dose.	Time.	Type of tumour.
Oberling and Guerin (1933)	Rats	Intraperitoneally	1 c.c., 10 inj.	After 9 months	Spindle cell sarcomas.
Roussy, Oberling and Guerin (1934)	,,	Intraperitoneally and subcutaneously	1 c.c. 5 inj.	Between 10th and 17th month	,, ,, ,,
Roussy, Oberling and Guerin (1936)	Rats and mice	Ditto	0·5 c.c.	Over 1 year	,, ,, ,,
Andervont and Shimkin (1940)	Mice	Subcutaneously	0·2 and 0·8 c.c.	,, ,, ,,	Spindle cell sarcomas and haemangiosarcoma.
Selbie (1938)	Rats and mice	,,	0·3 and 0·1 c.c.	Over 10 months	Spindle cell sarcomas and fibroma.
Onufrio (1938)	Rabbits	Intravenously	6 c.c.	2 years	Reticulum cell sarcoma.
van Mervennee and Then Thije (1939)	,,	Intravenously (?)	?	2 to 3 years	Sarcoma of spleen.
Foulds (1939)	Guinea-pig	Subcutaneously (mammary region).	0·3 c.c.	3 years	Carcinoma and sarcomata.

and spleen parenchyma with early and late degenerative changes, and (4) the fact that it is a radioactive substance and undoubtedly has dangerous cumulative effects.

Foulds (1939) obtained 1 carcinoma and 3 sarcomata in 20 guinea-pigs, 3 years after the injection into the mammary region of 0·3 c.c. of thorotrast, repeated each month for 4 months. The carcinoma and 2 of the sarcomas were transplantable.

Andervont and Shimkin (1940) studied the action of thorotrast in Strain A mice which have a high incidence of lung tumours. They concluded that the drug did not increase the incidence of lung tumours in this strain of mouse following intravenous injection of 0·2 to 0·8 c.c.

The first report of a possible connection between thorotrast and malignancy in man was made nearly 20 years after the introduction of the drug for clinical use. In 1947 McMahon, Murphy and Bates published the case of a woman aged 70 who had received 75 c.c. of thorotrast by intravenous injection (three doses of 25 c.c.) 12 years before for diagnostic purposes. She had at this time a gumma of the liver, which was treated, and she made a complete recovery. Twelve years later she developed weakness and pallor and died some time later with the diagnosis of " shock, the result of internal haemorrhage ". Three lesions were demonstrated histologically : ((1) Primary malignant liver tumour with metastases composed of cells resembling Kuppfer cells, which tended to arrange themselves in clusters, as walls of irregular blood sinuses or as walls of blood vessels replacing normal endothelium. Haemorrhage was a very prominent picture in the tumour. (2) Very heavy deposition of thorotrast with severe damage to the liver parenchyma. (3) Scars of healed syphilitic hepatitis with gumma formation. Although the association between tumour and thorotrast appears well demonstrated, the presence of chronic inflammation (syphilis) in the same organ makes it difficult to accept conclusively the aetiological rôle played by the thorotrast in this particular case.

Since this report, five other cases have been published in which thorotrast was thought to be the cause of the tumours found, as follows.:

Zollinger (1949) reported a case of a man of 48 who received 30 c.c. of thorotrast for a retrograde pyelography. Sixteen years later he developed a severe abdominal pain and a spindle cell sarcoma was discovered at operation, in relation to thorotrast deposits. Although Silva Horta (1953) accepts this tumour as definitely due to the action of thorotrast, we rather doubt if the hydronephrosis and chronic pyelonephritis which were the primary disease can be entirely ruled out as conditioning factors.

Rudolphi (1950) published a paper in which he described the case of a man who developed a squamous cell carcinoma of the right lower eyelid 35 years after receiving a preparation of thorium dioxide (0·27 g.) suspended in paraffin, for outlining the lachrymal duct. Chronic inflammation (giant cells) and granulation tissue were found associated with the growth, again bringing doubt about its possible rôle in the genesis of these tumours, although in this case the reaction appears to have been elicited by the presence of the preparation injected.

Abrahamson, O'Connor and Abrahamson (1950) described a lung tumour " with many exceptional, even unique features in a patient who 16 years previously had been injected with 75 c.c. of thorotrast ". Almost every alveoli in both lungs was lined by a tall secretory epithelium yielding mucin into the alveolar space and frequently throwing papillary projections of epithelium into the space. Metastases were found in the hilar lymph nodes and in the pancreas. The authors assumed they were dealing with a primary alveolar tumour of the lung of mucus

secreting type. Radio-activity was detected in the spleen and liver, but not in the lungs. This finding makes it extremely difficult to accept thorotrast as the causal agent of the tumour in question. Furthermore, it is worth while to note that the patient had respiratory troubles (breathlessness on exertion and cough) two years before thorotrast was given and that the histology of the tumour is in keeping with a slow development.

Silva Horta (1953) described a sarcoma of the liver in a woman who received 20 c.c. of thorotrast for cerebral angiography. She died 3 years and 2 months later, and the tumour in the liver had many similar features to that described by McMahon, Murphy and Bates (1947), and to one which we found in one of our animals (see below). The tumour was considered to be an endothelial cell sarcoma. No metastases were reported and degeneration of the liver parenchyma is referred to without any detailed description being given. Thorotrast was found associated with the growth.

The latest report on the subject came out in 1954. Matthes (1954) described a primary liver carcinoma found 21 years after intravenous injection of thorotrast for the purpose of hepatolienography. Lymph node metastases were reported.

These two latter cases appear to us to be the only ones in which there is a very strong case for thorotrast being the causal agent of the malignancies reported. Chronic inflammatory conditions were present in the other three cases and in the case of Abrahamson, O'Connor and Abrahamson (1950) a definite relationship between thorotrast and tumour was not demonstrated. In spite of these objections, however, a causal relationship between thorotrast and the tumours reported remains a possibility which is supported by the experimental investigations.

Since writing this review further work on " histological changes in man and rabbits after parenteral thorium administration ", has been presented by Dr. Charles Johansen at the Liége Symposium on Radiobiology, 1954.

PART II.

Report of Experimental Work.

The following is the report of an investigation on the effect of intravenously administered thorotrast in Schofield albino mice, with and without additional whole body X-irradiation. This experiment, when started, by two of us (L. F. L. and W. R. C.), was intended to be only of a preliminary nature and it was not planned to carry out detailed histological studies. However, the tissues from a number of the thorotrast-injected mice were kept, and the opportunity later arose for histological work (J. P. G.). The survival data in this experiment is not quite complete since unfortunately the record of death of several of the mice is missing, but it was felt, in view of the interest of the histological findings on the thorotrast-injected animals, that a report should be presented before the completion of a further more detailed experiment involving histological and autoradiographic studies of groups of animals killed at intervals, which is now under way.

Plan of the experiment.

Four groups, each of approximately 20 male Schofield albino mice, were used. The age of the mice at the commencement of the experiment was about 8 weeks. The four groups were treated as follows :

Group A : Control.

Group B : Injected intravenously with thorotrast (0·1 to 0·5 ml.).

Group C: Injected intravenously with thorotrast (0·1 to 0·5 ml.) plus 250 r whole body X-irradiation following the injection.

Group D: Given 250 r whole body X-irradiation.

The diet was exclusively Aberdeen rat cake. The four groups of mice were kept together in the same animal room in boxes containing 5 animals. Mice which became very ill were killed. An autopsy was performed on nearly all mice which were killed in the course of the experiment or which died naturally and in which *post-mortem* change had not proceeded too far.

The irradiation was carried out at 220 kV. (half-value layer 1·5 mm. Cu) at a dosage-rate of 11 r/min.

An attempt was made at first to inject each mouse with 0·5 ml. thorotrast. This proved immediately fatal in some cases and the dose was therefore reduced. In each Group B and C there were three dosage levels, 0·5 ml., 0·3 ml., and 0·1 ml.

The thorotrast used was an aged sample, having been prepared about 20 years previously.

Survival data.

Table III gives data on the survival of the mice in the different groups up to 15 months.

TABLE III.—*Survival Times in Control and Treated Mice.*

Group.	Treatment.	Number of animals.	Number alive at 15 months.	Times of death occurring within 15 months.
A	Control	20	9 (45%)	2, 3, 3, 4, 9, 10, 10, 15 months (not recorded in 3 cases).
B	Thorotrast:			
	ml.			
	0·5	5	3	3, 13 months.
	0·3	5	4	3 months.
	0·1	10	8	1, 13 months.
		20	15 (75%)	
C	250r + thorotrast:			
	ml.			
	0·5	5	2	6, 10, 12 months.
	0·3	6	2	15 days, 7, 13 months (1 not recorded).
	0·1	11	3	3, 6, 7, 8, 10, 10, 13 months. (1 not recorded)
		22	7 (32%)	
D	250 r	21	10 (48%)	2, 4, 5, 6, 8, 9, 11, 11, 12, 14, 15 months).

The number of animals involved is small and unfortunately the record of deaths is not quite complete, but the figures as they stand do not suggest that the injection of thorotrast in the doses given increases mortality within a period of 15 months.

Histological study of the thorotrast-injected animal.

The following report is based on a detailed histological investigation of 12 of the mice which received thorotrast intravenously (without whole body X-radiation)

Of the 20 mice in Group B given thorotrast, 3 died within the first 3 months. Seventeen animals lived more than 12 months and the last one was killed at 21 months after the injection. Tissues from 12 of these 17 animals were available for histological examination. All of these animals showed macroscopic change in various organs on autopsy. Systematic analysis of liver, spleen, lungs and kidneys was undertaken and in some cases the adrenal glands, lymph nodes, testis and bone marrow were also examined.

The 20 control mice of Group A were kept together with those injected, and 11 lived for more than one year. No histological examination was carried out on these controls, but macroscopically no tumours were found in any organ. Ten other mice three months old were carefully examined and none showed any pathological changes.

Sections were routinely stained by haematoxylin-eosin. Special staining methods such as Masson's trichromic, periodic acid—Schiff's fuchsin—Gordon and Sweet for reticulum, phloxin-tartrazine for inclusion bodies and Feulgen reaction were used when thought necessary.

The thorotrast particles are extremely easy to detect in the tissues on account of their refractive index. With haematoxylin-eosin they take a weak purple-brownish colour, staining brilliant green with Masson's trichrome (light green) and red with the periodic acid—Schiff's reagent.

Liver.—The distribution of thorotrast in the liver followed a fairly constant pattern. Most of the particles were found around the vessels (central and portal veins), mainly in clumps inside the cytoplasm of macrophages or less frequently lying free in the connective tissue of the portal spaces. Along the sinuses the material was chiefly seen in the cytoplasm of the Küppfer cells and rarely free in the lumen. We found a fairly uniform distribution of the material throughout the organ and no areas completely free of it were observed except in the case of hepatomas. Occasionally particles were seen in the cytoplasm of the liver cells. The presence of thorotrast was frequently found to be associated with degenerative changes in the liver cells, but the intensity of these changes was not always proportional to the amount of thorotrast present. An important finding was the complete absence of thorotrast within the hepatomas, being restricted to the parenchyma bordering the nodules.

Varying from animal to animal, but always present, peculiar degenerative changes were observed in the liver cells (Fig. 1). In the case of liver tissue from animals sacrificed at more than 6 months after injection, the nuclei were often enlarged, sometimes giant and bizarre, with an increased amount of chromatin, arranged in coarse granules intensely stained and irregularly distributed, an appearance which might possibly be due to polyploidy. The nuclear membrane was well marked and some distorted nuclei had a "potato-like" shape. As a rule the cytoplasm was abundant, faintly acidophilic and finely vacuolated, the limits between the cells being lost. An intra-nuclear body associated with these degenerative changes (Fig. 3) was practically always seen, in some cases being found in nearly every cell. The body was round in shape when small, and its size varied from very tiny to a large distorted structure sometimes occupying the whole nucleus. It had a basophilic, Feulgen positive rim, surrounding a granular, fuchsinophilic and Feulgen negative substance. Sometimes as many as five of these structures were found inside one nucleus. It is possible that these bodies are a type of nucleolar degeneration associated with metabolic changes in the liver

cells. Wilson (1953 and 1954) observed the same type of degenerative changes in the liver of mice kept on a diet containing bentonite (base-exchange silicate) which were followed by appearance of hepatomas.

From the present studies it appeared that the origin of these bodies could be traced back to the chromocentres, which in part would explain the great number of them sometimes found. They start as small, round structures, with a thick basophilic rim and a dark purplish interior substance. As they grow bigger, the rim gets thinner and the interior substance becomes progressively more scarce and weakly stained. Frequently the larger bodies show no acidophilic material, looking like irregular vacuoles in the nuclei. In all stages these structures were phloxin negative.

Vascular dilatation and some oedema of the Disse spaces were constantly seen (Fig. 1). Disruption of the trabecular arrangement and necrosis of the liver cells was a very rare event and no significant proliferation of the connective tissue was detected. A picture of cirrhosis was never seen in our animals. Despite those pronounced cellular alterations, the organs kept their normal general architecture fairly well and a lobular pattern could always be demonstrated.

A constant, and sometimes very prominent, proliferation of the reticulo-endothelial cells (Fig. 5) was seen in the majority of the animals examined, showing a definite increase in their phagocytic activity.

Five animals exhibited tumour-like masses (hepatomas) in the liver, which in one case showed atypical histological characteristics suggesting the possibility of malignancy (Fig. 2). These hepatomas closely resembled those obtained experimentally through feeding mice and rats with such substances as butter yellow, *Senecio jacobaea*, etc. In the present study the nodules observed were of different sizes and entirely non-architectural. Sometimes they were sharply separated from the rest of the parenchyma, which was compressed, while in others no clear separation could be traced. As a rule the cells were more basophilic than the normal and the nuclei smaller and more darkly stained. The abnormal nuclei described above were never found in these nodules. Some tumours showed the cells arranged in cords tending to radiate from centrally located vessels and separated by sinusoids, giving the appearance of normal parenchyma. However, there were no portal spaces related to these structures and a lobular pattern could not be seen. The more atypical nodules were composed of thick, finger-like columns of cells, invested by a layer of endothelial cells and separated by wide vascular channels. Alveolar arrangement and duct-like formations were often seen in these cases. Fatty degeneration was extremely pronounced in one case, which also showed large areas of haemorrhage. Necrosis was occasionally seen, being very extensive in two cases. No giant cells of any kind were observed and mitoses were extremely rare. No metastases were seen.

Associated with these hepatomas were varying numbers of intracytoplasmic bodies (Fig. 4), which have been previously described by Burns and Schencken (1940) in association with spontaneous and experimentally induced liver tumours. They were found predominantly at the periphery of the nodules and the larger ones had an internal refractile ring. They were consistently fuchsinophilic and Feulgen negative, and stained strongly with phloxin. No bile-duct proliferation or appreciable increase in connective tissue was seen associated with the nodules. However, no capsular reaction could be demonstrated, no definite invasion of the parenchyma could be clearly seen. The constant absence of thorotrast in the

mass of the nodules suggests that they grow by pushing out the surrounding tissues.

In one animal a malignant reticulo-endothelioma (malignant histiocytoma) was found (Fig. 6, 7 and 8). The sections examined showed a diffuse atypical proliferation of the reticulo-endothelial cells, exhibiting a remarkable phagocytic activity mainly with regard to the red cells. Areas of more compact growth showed spindle cells closely arranged, often lining clear sinusoid-like spaces. Collagen fibres were abundant in these areas and the reticulum was seen in a basement membrane-like position as regards the sinusoid formations. Invasion of the blood vessels was a common finding. Necrosis and haemorrhage were rarely seen and, surprisingly, mitosis was virtually absent. The liver parenchyma showed the degenerative changes already described, being also compressed and atrophied by the growth. Hepatomas of the nature described above were seen in the same liver. Thorotrast was found associated with this neoplasm. Extensive metastases in the kidneys and lungs were detected, showing chiefly a spindle celled structure. The tumour appeared to belong to the group studied some time ago by Gorer (1946).

Lungs.—The amount of thorotrast detected in the lungs varied with the dose given, but there was also a considerable individual variation among the animals. The particles were found mostly as emboli in the lumen of the capillaries and sometimes in the cytoplasm of the alveolar cells and phagocytes. No degenerative changes were observed in the pulmonary parenchyma and no inflammatory reaction associated with the thorotrast could be detected. Seven animals which died more than 15 months after the injections showed lung tumours. The tumours were multiple, mainly sub-pleural, and histologically resembled the common spontaneous and experimentally induced lung tumours in mice.

Since the days of Tyzer, (quoted by Stewart, 1953), controversy about the denomination, structure and origin of these tumours has existed and probably will go on as long as a dynamic process like histogenesis is studied by means of sections taken from different animals in different periods of time and the idea of growth and sequence of events built up from morphological pictures. There are two opposing views in this field. In one the tumours are believed to arise from alveolar cells, the involvement of the bronchi being a secondary event. In the other view the tumours arise in the bronchi and grow down invading the alveoli. We support a third view in which the terminal portions of the respiratory tree, both alveoli and bronchioles, are believed to take active part in the growth. In some of our tumours two types of cells could be clearly detected (Fig. 11), one being represented by a polygonal or round acidophilic cell, with small, dark, centrally located nucleus (alveolar cell), the other by a columnar cell, with granular and more basophilic cytoplasm, sometimes ciliated and a basal vesicular nucleus (bronchial cell). Occasionally mucus-like substance could be detected in the cytoplasm of the latter type. The relative proportions of these two types of cells is extremely varied and some tumours are almost exclusively formed by a single type (Fig. 13, 14 and 15). In the tumours or areas where the bronchial type of cells is predominant, tubular and glandular-like structures and papillary formations are the prominent pictures (Fig. 12, 16). The alveolar cells form more compact tumours, resembling collapsed alveoli with their walls lined by polygonal acidophilic cells. Smooth transition between the two types of cell could be detected in some tumours (Fig. 11). It is true, however, that in certain tumours no clear definition of the cell type could be detected, being a combination of the two types already described. Probably the

EXPLANATION OF PLATE.

FIG. 1 (*Animal No.* 4).—Abnormal nuclear pattern exhibited by the liver cell. Vascular dilatation. Proliferation of the reticulo-endothelial cells. Deposits of thorotrast about vessels. (H. & E. stain.) × 85.

FIG. 2 (*Animal No.* 2).—Liver. Cells arranged in wide finger-like cords and alveoli, invested by a layer of endothelial cells and separated by wide vascular channels. The picture is that of a hepato-cellular carcinoma. No thorotrast is seen. (H. & E. stain.) × 105.

FIG. 3 (*Animal No.* 4).—Liver cells exhibiting nuclear changes described in the text. The difference in size and shape of the intranuclear bodies and the finely vacuolated appearance of the cytoplasm can be seen. There is also some proliferation of the reticulo-endothelial cells. Particles of thorotrast are seen in the cytoplasm of the liver cell. (Feulgen stain.) × 600.

FIG. 4 (*Animal No.* 8).—Trabecular hepatoma. Intracytoplasmic "inclusion bodies" can be clearly seen. Neither nuclear aberrations nor thorotrast present. (H. & E. stain.) × 300.

FIG. 5 (*Animal No.* 11).—Liver. Hyperplasia of the reticulo-endothelial cells associated with deposits of thorotrast. (H. & E. stain.) × 390.

FIG. 6 (*Animal No.* 1).—Liver. Reticulo-endothelioma (histiocytoma). Area of solid growth showing spindle cells lining clear, sinusoid-like spaces. Deposits of thorotrast can be seen associated with the tumour cells. (H. & E. stain.) × 85.

FIG. 7 (*Animal No.* 1).—High power of Fig. 6. (H. & E. stain.) × 400.

FIG. 8 (*Animal No.* 1).—Liver. Showing the reticulum associated with the tumour cells and in a basement membrane-like position in regard to the clear spaces. (Reticulum stain.) × 110.

FIG. 9 (*Animal No.* 5).—Lung. Focus of bronchial and alveolar cell proliferation associated with deposits of thorotrast. No inflammatory reaction is present. (H. & E. stain.) × 70.

FIG. 10 (*Animal No.* 12).—Lung tumour. Possibly a later development of changes shown in Fig. 9. (H. & E. stain.) × 70.

FIG. 11 (*Animal No.* 12).—Lung tumour. High power of Fig. 10. Zone of transition between two types of cells, (*a*) polygonal or round cells, sometimes with a vacuolated cytoplasm and with a dark, small and centrally located nucleus; (*b*) columnar cells with a granular cytoplasm and a vesicular, basally located nucleus. One type changes smoothly into the other. (H. & E. stain.) × 110.

FIG. 12 (*Animal No.* 12).—Part of the tumour shown in Fig. 11 and 15 giving more details of the bronchial type cell component. Inflammatory exudate is present inside some of the tubular structures. (H. & E. stain.) × 110.

FIG. 13 (*Animal No.* 12).—Tumour found in the other lung of the same animal (Fig. 12). The tumour is entirely composed of alveolar cells. Respiratory and terminal bronchioles are apparently not taking part in the growth. (H. & E. stain.) × 80.

FIG. 14 (*Animal No.* 12).—Lung tumour. Lower power of Fig. 13. (H. & E. stain). × 22.

FIG. 15 (*Animal No.* 12).—Lung tumours. Higher power of Fig. 14 showing alveolar cells desquamating and exhibiting phagocytic activity. (H. & E. stain.) × 450.

FIG. 16 (*Animal No.* 12).—Lung tumour. Another field of the tumour already shown in Fig. 12. Associated with the papillary growth an atypical connective tissue proliferation can be seen, containing areas of necrosis. (H. & E. stain.) × 22.

FIG. 17 (*Animal No.* 12).—High power of the connective tissue shown in Fig. 16, showing the abnormal nuclear patterns. (H. & E. stain.) × 360.

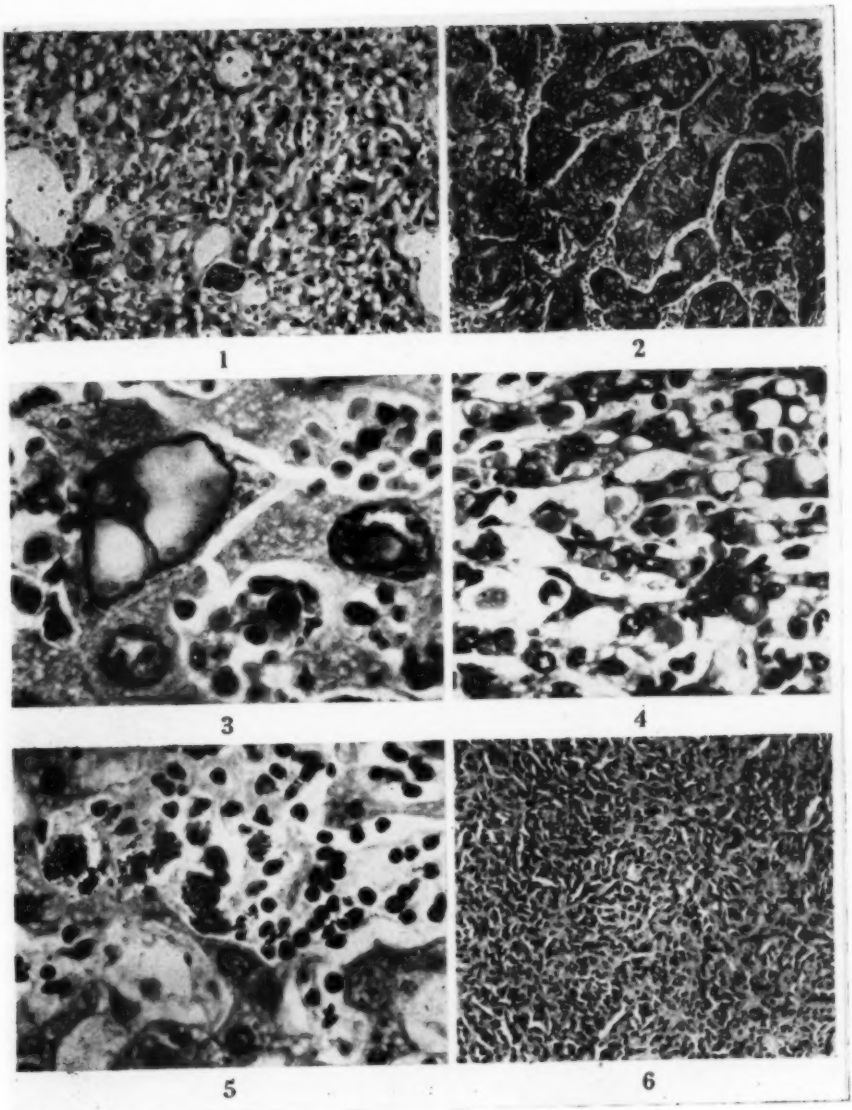
FIG. 18 (*Animal No.* 8).—Spleen. Thorotrast collected in the macrophages of the pulp in an otherwise normal organ. (H. & E. stain.) × 80.

FIG. 19 (*Animal No.* 3).—Lung tumour. Intrabronchial and sub-pleural growths of same histological pattern apparently unrelated. Some perivascular infiltration. (H. & E. stain.) × 25.

FIG. 20 (*Animal No.* 3).—Lung tumour. High power of the intrabronchial growth shown in Fig. 19. The papillary formations apparently arise from the bronchial wall. (H. & E. stain.) × 450.

FIG. 21 (*Animal No.* 5).—Spleen. Vascular haemangio-endothelioma. Deposits of thorotrast can be seen. (H. & E. stain.) × 125.

FIG. 22 (*Animal No.* 5).—Liver. Same animal as Fig. 21. Metastases from the haemangio-endothelioma. (H. & E. stain.) × 125.



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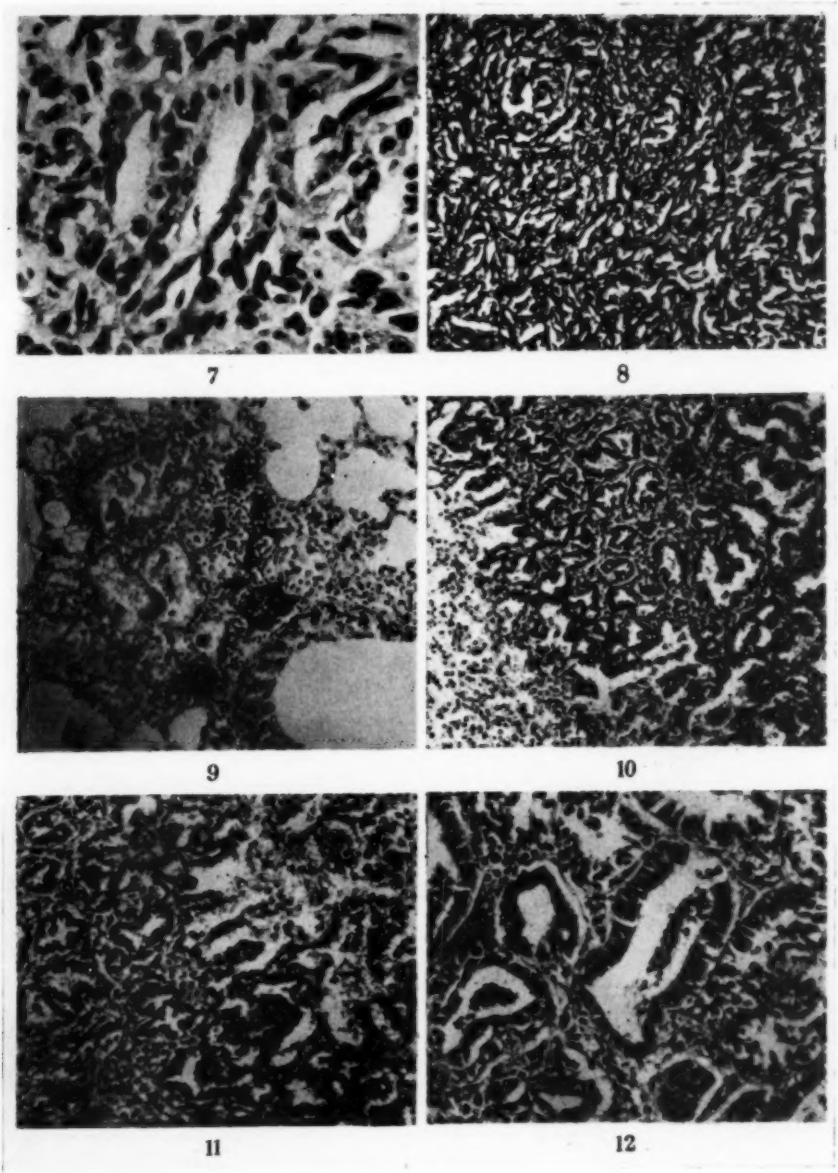

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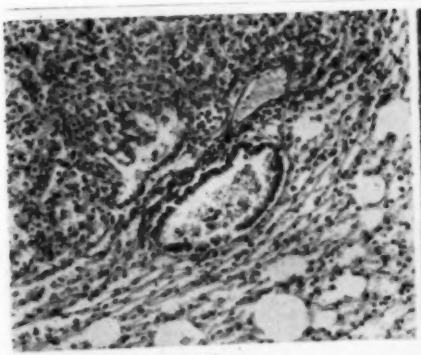

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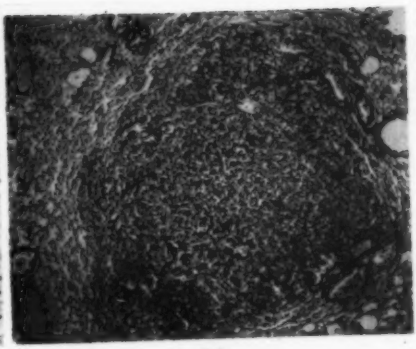

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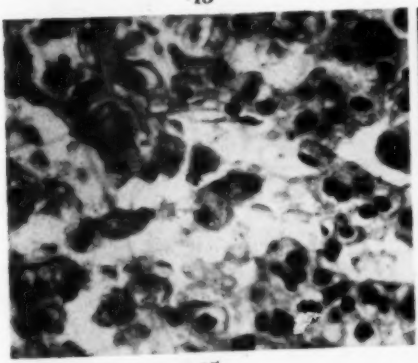

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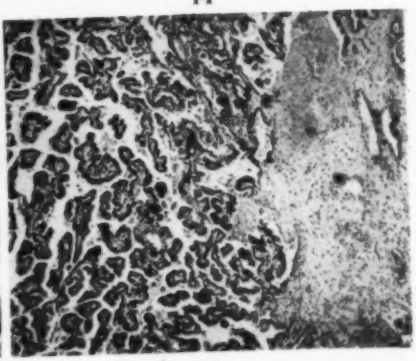

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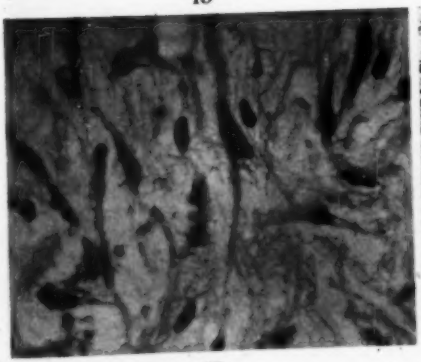

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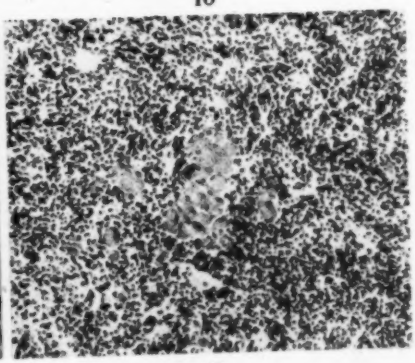

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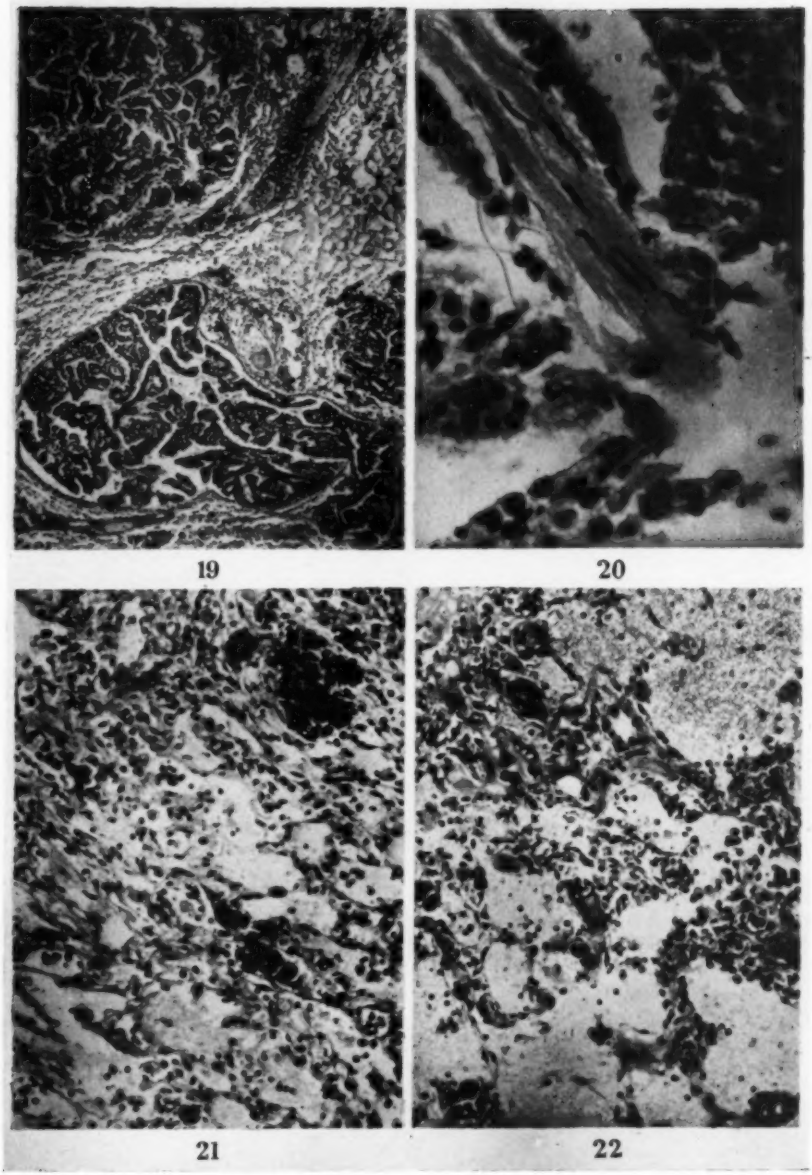

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TABLE IV.—*Summary of Pathological Findings in Thorotrast-Injected Mice.*

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Animal.	Dose (c.c.).	Death after injection (months)	Liver.	Spleen.	Kidneys.	Lungs.	Presence of thorotrast.
1	0·5	13	Liver cell degeneration. Nuclear giantism. Hepatomas. Reticulo-endothelioma	Slight fibrosis	Metastases of the reticulo-endothelioma	Oedema and haemorrhages. Metastases of the reticulo-endothelioma	Abundant in liver and spleen. Moderate in kidneys and lungs.
2	0·5	17	Moderate liver cell changes. Reticulo-endothelial proliferation. Hepatomas.	*Post-mortem* changes (haemangio-endothelioma)	Columnar metaplasia of the capsular epithelium	Tumours	Abundant in liver, lungs and spleen. Moderate in the kidneys.
3	0·5	21	Liver cell changes. Nuclear giantism. No tumours	Ditto	Ditto	„	Abundant in liver and spleen. Appreciable amount in kidneys and lungs.
4	0·3	18	Ditto	N.A.	N.A.	Tumour	Ditto.
5	0·3	20	Liver cell changes. Hepatomas. Metastases of haemangio-endothelioma (?)	Haemangio-endothelioma	„	Tumour (first stages ?)	Abundant in liver and spleen. Appreciable amount in the lungs and kidneys.
6	0·1	13	Liver cell changes. Hepatomas. Fatty degeneration and extensive necrosis	N.A.	„	No tumours	Abundant in liver and spleen. Very small amount in lungs and kidneys.
7	0·1	15	Moderate liver cell changes. No hepatomas. Diffuse reticulo-endothelial hyperplasia	Slight fibrosis and increased cellularity of the pulp.	„	„ „	Abundant in liver moderate amount in kidneys, lungs and spleen,
8	0·1	17	Moderate liver cell changes. Hepatomas	N.A.	„	„ „	Abundant in liver and spleen. Moderate in kidneys. Practically no THO_2 in the lungs.
9	0·1	18	Liver cell changes. No hepatomas. Reticulo-endothelial hyperplasia	Some increased haemopoietic activity	Some metaplasia of the glomerular epithelium	Tumour	Abundant in liver and spleen, Extremely scarce in lungs and kidneys.
10	0·1	18	Liver cell changes. No hepatomas. Some reticulo-endothelial proliferation	Slight fibrosis	N.A.	„	Abundant in liver and spleen. Scarce in lungs and kidneys.
11	0·1	18	Liver cell changes. No hepatomas. Prominent reticulo-endothelial proliferation	Slight fibrosis	„	Tumours	Abundant in liver and spleen. Small amount in lungs and kidneys.
12	0·1	21	Liver cell changes. Reticulo-endothelial proliferation	N.A.	„	„	Abundant in liver and spleen. Minimal in lungs and kidneys.
Total tumours			5 hepatomas 1 reticulo-endothelioma	1 (? 3) haemangio-endothelioma	No tumours	7 lung tumours	

neoplastic proliferation of both alveolar and bronchiolar cells tends to a unique type of cell, on account of their embryological relationship. Localized or diffuse hyperplasia of the epithelium of the bronchioles and alveoli appeared to precede the tumour formation and in a few cases a definite association between the presence of thorotrast and proliferation was seen (Fig. 9). The tumours appeared histologically benign. In one case a tumour was of considerable size, invading the surrounding tissues, and areas of necrosis were present. This tumour showed also an area of atypical proliferation of connective tissue associated with the epithelial growth (Fig. 16, 17). Mitoses were extremely rare and metastases were not seen in any cases.

Spleen.—The distribution of the thorotrast in the spleen followed the pattern described elsewhere by other authors. Almost the whole amount of thorotrast was found in the cytoplasm of the macrophages, either in the pulp or in the sinuses (Fig. 18). Very few particles were seen lying free in the tissues. The Malpighian bodies were comparatively free of thorotrast, although small particles could be traced in the cytoplasm of macrophages. Some increase in size of the Malpighian bodies and varying trabecular fibrosis were the most constant findings. Degenertive changes were not observed.

In one animal a malignant haemangio-endothelioma was found (Fig. 21). The normal architecture of the organ was entirely lost, being replaced by a tumour composed of vascular channels, varying in diameter from a capillary to a large blood sinus, lined by abnormal swollen endothelial cells which were proliferating actively and desquamating into the lumen. Areas of necrosis and haemorrhage were seen and at one point the capsule was ruptured. At the *post mortem* examination the peritoneal cavity was found to be full of blood. The same histological picture was found in two areas in the liver, suggesting metastases (Fig. 22), although the possibility of a multifocal tumour could not be entirely ruled out. Heavy deposits of thorotrast were seen associated with the growth. Two other animals which showed blood in the peritoneal cavity exhibited an enlarged spleen with extensive areas of haemorrhage. Unfortunately advanced *post-mortem* changes made it impossible to reach any definite diagnosis although in at least one case the possibility of a haemangio-endothelioma was strongly suggested.

Kidneys.—The amount of thorotrast in the kidneys was much smaller than that found in the liver, spleen and lungs. It was seen in the interstitial tissue, collected as small particles in the cytoplasm of macrophages, or as emboli in the capillaries of the stroma. Rarely was it seen in the capillaries of the glomeruli. Particles of thorotrast were never observed either in the epithelial cells or in the lumen of the tubules.

In certain cases adrenal glands, bone marrow and testicles were examined, but nothing significant was detected.

Comments.

Although no indisputable proof can be produced that our tumours have been induced by thorotrast, the constant association between particles and growth, the occurrence of relatively rare tumours of liver and spleen and the absence of neoplasms in the control group strongly suggest a causal relationship.

The case of the lung tumours is particularly interesting in view of the importance of the association between lung cancer and radiation, (Hueper, 1942; Lorenz, Heston, Deringer and Eschenbrenner, 1946).

In the present investigation lung tumours were observed in mice examined more than 15 months after the injection and all of them were macroscopically recognised as small whitish nodules on the surface of the organ.

Areas of epithelial proliferation (bronchial and alveolar), apparently the first stages of tumour formation, were seen associated with deposits of thorotrast, supporting the view that radiation was directly responsible for a proliferative activity.

A search of the literature has yielded only very few reports on the relationship between radiation and liver tumours. Besides those neoplasms found in humans treated by thorotrast, reports on only three experimental observations have been found. Daels and Biltris (1931) obtained three cholangio-cellular carcinomas in guinea-pigs by means of radium sulphate. Petrov and Krotkina (1933) produced hepatocellular carcinomas with metastases in guinea-pigs by introducing radium into the gall bladders. Lacassagne and Joliot (1944) found a primary liver carcinoma in a rabbit which had been exposed to bombardment by neutrons for three days and died nine and a half months after exposure. Spontaneous cancer of the liver in rabbits being unknown, the tumours was attributed to the radiation.

One view regarding the pathogenesis of the experimental liver tumours maintains that they are the end-product of a continued sequence of destruction and regeneration of the liver cells. In our cases, however, a picture of cirrhosis with its three components—nodular hyperplasia, fibrous and bile-duct proliferation —which is the accepted outcome of prolonged damage to the liver parenchyma, and is an intermediate step to the great majority of liver tumours experimentally induced, was never seen. Furthermore, in some cases a smooth transition was clearly seen between degenerated cells and hepatomas.

As previously mentioned, no histological examination of the control animals which lived over 12 months was made in this experiment and the degenerative changes in the liver cells could not therefore be checked. However, it should be noted that animals which died in the first months after the injection did not show any of the degenerative changes described above, and that 10 animals, about 3 months old, thoroughly examined histologically, failed to reveal any liver change. These facts suggest that the changes were brought about by the prolonged action of the thorotrast, although the possibility of additional factors, for instance, nutritional factors, cannot be ruled out. It is interesting to note that in the experiments of Wilson (1953), when mice were fed with bentonite, 8 animals out of 11 which developed hepatomas had received thorotrast previously for X-ray purposes. However, the authors claim that 18 control animals, which lived 216 to 511 days after the injection of thorotrast, did not show any liver tumours.

The reticulo-endothelioma found in the present study appeared to be preceded by an active reticulo-endothelial proliferation, which was constantly seen in other animals of the same group, reaching in some cases great prominence. The virtual absence of mitoses in a tumour of such malignant behaviour was remarkable. The findings of one (possibly three) malignant haemangio-endotheliomata of the spleen was highly interesting on account of the rarity of primary splenic tumours of this type.

The views regarding the mechanism of the neoplastic changes induced by radiation can be reduced, essentially, to two : (i) the malignancy is induced by direct cellular stimulation either by means of chromosome damage or by disrupting the normal cellular metabolic pattern ; (ii) the malignancy appears as an end-

product of a sequence of non-specific destructive changes in the irradiated tissue alternating with attempted regeneration. The fact that chronic irradiation with small doses appears to be more effective in inducing tumours than large single doses, at least as regards certain tumours and species, and that some tumours are preceded by chronic inflammation (radio-osteitis before ostoegenic sarcoma, radio-dermatitis before skin carcinoma, etc.) seems to support the second view or at least to suggest that radiation-induced tumours are a result of previous slow cumulative changes.

An important experiment on this subject was carried out by Glucksman (1951). He delivered a large single dose of β-rays to the skin of rabbits in order to compare the changes induced with those obtained by painting the skin with benzpyrene. In the irradiated area he observed cyclical stages of ulceration and healing lasting over one year, until tumours began to appear 14 months after the irradiation, arising in the reparative epithelium. The instability of the repair reaction was attributed to the vascular damage induced by the irradiation and the neoplastic changes to a probable adaptive variation brought about by a constant abnormal stimulus. The benzpyrene induced an immediate and direct hyperplastic epithelial reaction followed by neoplasia 3 months later. Although the explanation given by Glucksman (1951) appears to be the logical one, it might be assumed that the very fact of the primary incapacity to heal in itself indicates a directly induced irreversible change, the histologically recognisable tumours being the morphological manifestation of previous biochemical change.

Owing to the lack of information on so many aspects of the problem of radiation carcinogenesis, all comment is bound to be highly speculative. If one admits that thorotrast is the causal agent of the tumours observed in the present experiments, one is led to think of its action as directly upon the cells and not through unspecific destructive effects. The absence of cirrhosis of the liver and of pulmonary fibrosis gives no support to the latter possibility.

It is hoped that some of the outstanding questions raised by this study will be answered by the more detailed experiment now being carried out.

SUMMARY.

A survey of the literature on the late effects of thorotrast, with particular reference to carcinogenic effects, is given. A short report follows on the effects of intravenously administered thorotrast in mice, with and without the addition of whole body X-irradiation. Detailed histological findings in twelve animals which were given thorotrast intravenously are presented and discussed. Degenerative changes in the liver, five hepatomas, one reticulo-endothelioma of the liver, one haemangio-endothelioma of the spleen and seven lung tumours were found. Eleven control animals which lived over 12 months failed to reveal any tumour at a macroscopic examination.

This work was carried out jointly in the Physics and Radiotherapy Department of the Institute of Cancer Research, Royal Cancer Hospital, directed by Professor W. V. Mayneord and Professor D. W. Smithers. We are grateful to Mr. J. E. Gibbs for section cutting, to Miss M. M. Gwyther for photography, to Miss K. Adams and Miss M. Winsborough for valuable assistance, and to Miss M. P. Leach for preparation of the manuscript for publication. The financial assistance of the Medical Research Council, the British Empire Cancer Campaign and the National Research Council (Brazil) is gratefully acknowledged.

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THE SIGNIFICANCE OF THE SEQUENCE OF INITIATING AND PROMOTING ACTIONS IN THE PROCESS OF SKIN CARCINOGENESIS IN THE MOUSE.

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THE two-stage mechanism of carcinogenesis (see review : Berenblum, 1954*a*) provides the basis for a new approach to the problem of tumour pathogenesis. It postulates that a specific " initiating action " converts one or more normal cells into " dormant tumour cells ", and that the latter require, for their evolution into visible tumours, a " promoting action ", apparently involving a delay in maturation until a critical-size colony of dormant tumour cells is reached (Berenblum, 1954*b*). An essential feature of this system is that the respective mechanisms of the two stages must be different and independent, so that the completed process of carcinogenesis, which results when promoting action *follows* initiating action, should not occur when the procedure is reversed. [If carcinogenesis merely depended on a summation of two sub-optimal doses of (one strong and one very weak) carcinogen, or if it involved a summation of two actions possessing the same mechanism, e.g. two mutations, as implied in the theories of Fisher and Hollomon (1951), Arley and Iversen (1952), Nordling (1953) and others, then it should be immaterial which action came first.]

There is already some evidence in support of the dependence of carcinogenesis on a correct sequence of initiating and promoting actions—when croton resin (the active principle of croton oil) is applied to the skin for 26 weeks *before* a course of treatment with 3 : 4-benzpyrene, no speeding up of tumour production occurs, but when the croton resin is administered *after* a short course of benzpyrene treatment, carcinogenesis is greatly augmented (Berenblum, 1941*b*). Indeed, this observation was the starting-point of the studies on the promoting action of croton oil or resin (Berenblum, 1941*b*), which, in turn, provided a quantitative basis for the two-stage mechanism concept (Berenblum and Shubik, 1947, 1949).

However, to prove the point, it was necessary to carry out a more critical experiment—a comparison between tumour production resulting from a single application of a carcinogenic hydrocarbon (for initiating action) followed by 40 half-weekly applications of croton oil (for promoting action) and that occurring when the 40 half-weekly applications of croton oil are followed by a single application of the carcinogenic hydrocarbon. Since this had not previously been carried out, the present investigation was undertaken.

METHODS.

The mice used for this investigation were females of the Swiss strain, previously pen bred, and since their acquisition in these laboratories, by brother-to-sister

mating for 10–11 generations. They were fed on Purina Laboratory Chow (Rawlston Purina Co., U.S.A.) and water *ad libitum*, and kept in air-conditioned rooms at 21–22° C. At the start of the experiment, they were 3½ months old. The mice were entirely free from any known infection, and the mortality during the period of treatment (23 weeks) was negligible (2/42 deaths).

Before each application, the hair over an area of about 1 sq. cm., in the region of the shoulder blades, was carefully clipped with fine scissors. The solutions for application (1·5 per cent 9 : 10-dimethyl-1 : 2-benzanthracene or 5 per cent croton oil, both in liquid paraffin) were delivered by a glass rod. The resulting tumours were charted when first observed, and fortnightly thereafter. Papillomas that appeared in some cases on the ears were charted likewise.

RESULTS.

In the first group, 22 mice received a single application of DMBA (1·5 per cent 9 : 10-dimethyl-1 : 2-benzanthracene in liquid paraffin) to the skin of the back; the mice were then left untreated for 3 weeks, and thereafter received twice-weekly applications of croton oil (5 per cent in liquid paraffin) for 20 weeks, and then kept for a further 15 weeks for observation. The results of the experiment are summarized in Table I.

TABLE I.—*Development of Tumours in Mice Treated with Dimethylbenzanthracene and Croton oil.*

Treatment.		Mouse No.	First appearance of tumour (weeks).		Tumours.		
Primary.	Secondary.		(a).	(b).	Skin of back.	Ears.	Persistence.
DMBA × 1	Croton oil × 40	28–1	10	7	2 P	—	+
			14	11	—	2 P	+ +
		28–2	10	7	2 P	—	+ +
		28–3	10½	7½	—	6 P	+ +
			16½	13½	1 P	—	+/−
		28–4	11½	8½	4 P	—	+
		28–5	12	9	2 P	—	+/−
		28–6	12	9	2 P	—	+/++
			18	15	—	3 P	+ +
		28–7	14	11	1 P	—	+/−
			14	11	—	4 P	+ +
		28–8	14	11	1 P	—	+/−
		28–9	14½	11½	—	1 P	+/−
		28–10	14½	11½	1 P	—	+ +
		28–11	17½	14½	—	2 P	+ +
		28–12 to 28–22	—	—	—	—	—
		Total			16 P	18 P	
Croton oil × 40	DMBA × 1	29–1	16½	—	1 P	—	+ +
		29–2	25	2	1 P	—	+
		29–3 to 29–20	—	—	—	—	—
		Total			2 P	—	

(a) = from commencement of primary treatment; (b) = from commencement of secondary treatment; P = papillomas. + = persisting without growth in size; + + = progressive growth in size; +/− = regressed after 6 weeks or more; +/++ = original tumour disappeared but others appeared later.

Eleven of the 22 mice developed papillomas which were, in most cases, multiple: in 5 mice the tumours were confined to the treated skin (10 papillomas); in 4 mice they developed both in the treated skin (6 papillomas) and on the ears (15 papillomas); and in 2 mice they arose only on the ears (3 papillomas). The latent period ranged from 7–14½ weeks from the start of the second treatment (croton oil), or 10–17½ weeks if calculated from the beginning of the experiment. No new tumours developed after that, though the croton oil treatment was continued for another 5 weeks and the animals observed for a further 15 weeks. (This is in keeping with the earlier results of Berenblum and Shubik, 1947.) The persistence, growth, or regression, of the tumours, are indicated in Table I. (The recording of a tumour as "regressed" denoted that it had at least persisted for 4 weeks, and papillomas that disappeared in less than 4 weeks were not counted.)

In the second group the procedure was reversed: 20 mice received twice weekly applications of croton oil; they were then left untreated for 3 weeks, and then given a single application of DMBA, the dosage and other conditions of the experiment being otherwise identical with those of Group 1. These mice were also kept for observation for 15 weeks after cessation of treatment.

The results of this second group (also summarised in Table I) were strikingly different from those of the first group: only two of the 20 mice developed tumours, in each case a solitary papilloma. One of these appeared in 16½ weeks from the commencement of the experiment, i.e. before the second treatment was even begun; the other after 25 weeks from the start of the experiment, i.e. 2 weeks after the second treatment. In the one case the tumour grew progressively; in the other it remained stationary. No papillomas appeared on the ears in this group.

None of the tumours of either group became malignant.

DISCUSSION.

The results essentially confirm previous expectation on the basis of the two-stage mechanism of carcinogenesis. Tumours developed in large numbers when promoting action *followed* initiating action, but not when the procedure was reversed.

The two papillomas that developed in the second group can be readily discounted, since one of them arose even before the second treatment was given and the other only two weeks after the second treatment. Both must, therefore, be attributed to a weak carcinogen action of the croton oil itself, of which examples have been previously recorded in rare instances (Berenblum, 1941*a*; Salaman and Roe, 1953).*

Even if the second tumour were accepted, the result of this experiment would be: eleven out of 22 mice developed tumours when promoting action followed initiating action, while one out of 20 developed tumours when promoting action came first; or, if expresed in total number of tumours, 34 tumours developed in the first group and only 1 in the second.

SUMMARY.

1. A single application of 9 : 10-dimethyl-1 : 2-benzanthracene to the mouse's skin, followed by 40 twice-weekly applications of croton oil, led to the develop-

* At the 6th International Cancer Congress, Sao Paulo, Rusch (1954) reported on a strain of mice in which skin papillomas developed in larger numbers after croton oil treatment.

ment of papillomas in half the treated animals. When the treatment was reversed, with the 40 applications of croton oil *preceding* the single application of the dimethylbenzanthracene, only 2 out of 20 mice developed papillomas, of which one appeared before the second treatment was begun.

2. These results confirm the principle on which the two-stage mechanism of carcinogenesis is based, namely, that the two stages—initiating and promoting—represent different and independent processes which do not act by summation, but only when administered in a particular sequence, whereby the initiating action converts some normal cells into dormant tumour cells while promoting action converts the dormant tumour cells into recognizable tumour masses.

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POSSIBLE SIGNIFICANCE OF ABNORMAL DERMAL COLLAGEN AND OF EPIDERMAL REGENERATION IN THE PATHOGENESIS OF SKIN CANCERS.

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THE relative rôles of epidermis and of connective tissue in the histogenesis of skin cancers has been a subject of considerable controversy in the literature. After his careful study of the histopathology of carcinomas resulting from Roentgen irradiation, Wolbach, in 1909, arrived at the conclusion that the supervention of carcinoma in sites subjected to excessive radiation was due to dermal injuries, and especially to damage to the vascular elements in the cutis, which resulted in interference with the nutrition of the overlying epithelium. The interference with the nutrition of the epidermis, combined with the frequent existence, in sites of radiation injury, of fibrin thrombi and areas of dense, bloodless, collagenous material, according to Wolbach (1909) leads to :

> "A slow augmentation of the growth ... (of the epithelium) that finally results in the ability of the epithelial cells to derive their sustenance at the expense of other living tissue. ... The acquisition of malignant powers is completed during years of active proliferation accompanied by progressive impairment of nutrition."

This view, propounded by Wolbach, has since been supported by a number of students in this field, and more especially by Puhr (1935), Orr (1939), Brown, McDowell and Fryer (1949) and, more recently, by Vernoni (1951), and by Marchant and Orr (1953). The relevant literature has been critically reviewed by Orr, Vernoni, and by Marchant and Orr.

Nevertheless, there is a school of thought which maintains that the production of carcinoma (by various radiations and other injuries to the skin), is attributable to primary effects of the injurious agent on the epidermis (Montgomery, 1946 ; Teloh, Mason and Wheelock, 1950).

From previous analyses of the pathogenesis of hepatic cirrhosis and of primary carcinoma of the liver (Gillman and Gillman, 1951), it was suggested that the epithelial and connective tissue components, in the liver at least, were governed by separate regulations and that the ultimate histopathological picture encountered in a damaged liver was a reflection of the relative responses of each of these basic components. We have now accumulated quite a considerable body of evidence, from the study of human and animal material, to support this view for the skin as well as for the liver. Thus, during the healing of various types of skin

wounds, in animals and in man, we found that at one stage the epithelium was apparently the organiser, while at another the connective tissue responses, initially evoked by the regenerating epithelium, were in turn responsible for the final pattern of healing. We hope, below, to adduce further evidence to indicate that the reactions of the epidermis to injury may be profoundly influenced by the state of the related connective tissue.

Another controversial problem, in the field of carcinogenesis of the skin, relates to the site of origin of the carcinoma itself. In recent years much evidence has been accumulated to substantiate the view, originally propounded by Mallory (1914) and subsequently supported by the careful studies of Foot (1947) and Lever (1948*a*, 1948*b*) that skin cancers arise from hair follicles. Foot (1951) has recently reviewed newer evidence relating to this problem and considers it as clearly established that skin carcinomas should be regarded as " adnexal carcinomas " arising from " the *necks* of hair follicles where there is an abundance of poorly differentiated basal cells " and not, as originally maintained by Mallory, from an *adult* hair *matrix*. Wolbach (1951) in another of his scholarly studies, has provided strong experimental evidence from his work in mice to substantiate his view that : " Papillomas take origin from hair follicles that have lost their papillae cells." More recently, Chase and Montagna (1951), and Chase (1954), Andreasen (1953) and Andreasen and Engelbreth-Holm (1953) have presented further interesting data to demonstrate the close connection which exists between the physiological state of the hair follicles in mice and the epidermal responses to applied carcinogens.

All the work of the above-mentioned, as well as of many other experimental and human oncologists who maintain that skin cancers develop from hair follicles, is based on the assumption (hitherto regarded as fact) that there can be no regeneration of hair follicles from surface epithelium in adult animals. Wolbach (1951) states quite dogmatically that: "In the mouse, new follicles do not form from the epidermis, under any circumstances, after the original postnatal formation has been completed." However, in recent years, a considerable body of evidence has been accumulated to indicate that this statement by Wolbach, which summarizes generally maintained views on this subject, is not in conformity with fact, either for animals or for man. Thus, in 1953, we presented evidence that neogenesis, at least of sebaceous glands, could occur in man under certain circumstances. Subsequently, we have reported further that it seemed likely, both in man and in animals, that regeneration of hair follicles in adults could occur in association with wound healing. We were not in a position to *prove* this possibility conclusively. However, it has become apparent from our more recent studies that at certain stages of the repair of some types of wounds " pseudo-pegs " of epithelium were constantly found to invade the underlying connective tissue, and that this was a normal feature of the uncomplicated healing of wounds (Gillman *et al.*, 1953, 1955). Normally, however, these invasive spurs of epidermis, which in animals may closely simulate regenerating hair follicles, are eliminated, for the most part, apparently by virtue of the activity of regenerating connective tissue. We were glad, therefore, when Breedis (1954) recently reported the results of his elegant experiments, in which he has been able to establish, with considerable clarity and certainty, that regeneration of hair follicles and sebaceous glands from scar epithelium does in fact occur, at least in the experimental situations devised by him in rabbits.

This evidence for the existence of regeneration of hair follicles and sebaceous glands in adults carries considerable implications for further studies of the aetiology and histogenesis of skin cancer. Wolbach stated that :

> " Papillomas develop from hair follicles which have lost their papillae rest cells. . . . This conclusion was arrived at after failure to find these cells in any stage of papilloma formation, and with the knowledge that papillae cells may be destroyed by repeated applications, either following depilation at the quiescent stage, or in active follicles after the hair canal has been formed.
>
> " The follicle deprived of its papillae cells is no longer the source of new follicle formation."

We have shown that invasive spurs of epidermis appear normally during the healing of acute and chronic injuries to the skin. Whether or not these invasive spurs of epidermal cells will be eliminated, or develop into new skin appendages, or into benign or malignant neoplasms, will apparently depend largely on at least three circumstances, namely :

(1) The nature and extent of the injury to the two component tissues of the skin.

(2) The duration and frequency of such injury.

(3) The maintenance and disruption of those epithelial-connective-tissue relations which are maintained during the slow day-to-day regeneration, or during the much more rapid regeneration which supervenes after acute or chronic injury to the skin.

We propose here to present evidence to show :

(a) That epidermal " invasiveness " is a normal finding in healing wounds.

(b) That by altering the state of the connective tissues related to a healing wound, this epidermal invasiveness can be so distorted in human subjects as to result in a reaction closely simulating skin carcinoma.

Some possible implications of these findings for the study of carcinogenesis will be briefly discussed.

MATERIAL AND METHODS.

Material.

In a previous study (Gillman, *et al.*, 1953) we provided details of the experimental procedures adopted in a series of grafting experiments in human volunteers. Briefly stated, thin split skin (Thiersch) grafts were removed from the antero-medial aspect of the forearm and various types of grafts were replaced on the healing donor sites. The histogenesis of healing in the untreated and in the graft-treated donor sites were analysed from the microscopic study of repeated biopsies. Of particular interest for the present study were the reactions evoked by the application, to such healing Thiersch-graft donor sites, of homo-dermal tissue, prepared by the removal of the epidermis from split-skin homo-grafts, following trypsin digestion according to the method detailed by Billingham and Reynolds (1952).

Our original descriptions were based on biopsies taken from 5 European volunteers. We have since repeated this study, this time on five non-European volunteers. The present report on the reactions evoked by the application of trypsin-treated homo-dermal grafts to healing Thiersch-graft donor sites is thus

based on 69 biopsies taken from 10 human volunteers and including specimens removed from the first to the eightieth post-operative days.

In addition, we have used, for this study, specimens from 20 cases of skin lesions (including skin carcinoma) in man, induced by chronic ultraviolet, X-ray or radium injury to the skin. Many of these cases yielded large blocks of tissue, such as the entire skin covering the dorsum of the hand, which were excised because of the presence in these areas of multiple stages in the pathogenesis of skin cancers. Ten or more blocks of tissue were sometimes taken from such large specimens and treated as outlined below. Tissues were also available from a single case in whom a carcinoma had supervened, following a minor cut to a 27-year-old burn scar on the temple. Furthermore, sections of 40 scars of varying age and type, resulting from the healing of different sorts of injuries were studied.

While we are not reporting here, in detail, the information available from the study of skin sections taken from over 150 mice treated with 0·3 per cent methylcholanthrene in acetone, for varying times and under varying experimental conditions, nevertheless our findings have been used to clarify our understanding of the phenomena here described.

Methods.

All material was fixed in formalin, dehydrated through alcohols, embedded and sectioned, and then mounted serially as previously described. The most important stains applied to serial slides of the same specimen were : Haematoxylin and eosin, Weigert's Verhoeff's, and/or Taenzer-Unna's acid-orcein methods for elastic fibres, Mallory's triple connective-tissue stain, a modified Masson-method using Phloxine 2R and Light-Green, Mallory's phosphotungstic acid haematoxylin stain, as modified by Jackson (1951, personal communication), dilute toluidin-blue, Gomorri's aldehyde fuchsin, Periodic-Acid-Schiff (Lillie, 1952), a modified Hale's iron method for mucopolysaccharides (Rinehart and Abul Haj, 1951) and the original Safranin and polychrome methylene blue method described by Unna, and detailed by Hall and Herxheimer (1905).

OBSERVATIONS.

We have described in detail elswhere (Gillman *et al.*, 1953, 1955) the normal healing of untreated Thiersch-graft donor sites in healthy volunteers. It may be stated briefly here that during the first four to six post-operative days the epidermis regenerates and quickly covers the donor site. At this stage the new epithelium lies in direct contact with the denuded stratum reticularis of the dermis, and there is virtually no sign of connective-tissue regeneration. The latter commences between the fourth and seventh post-operative days, and increases progressively until about the fifteenth post-operative day, when the new connective tissue starts to become fibrosed. During this second phase (eighth to fifteenth days), the epithelium becomes markedly hyperplastic and numerous spurs, of various thickness and depth, seem to invade the new underlying connective tissue (Fig. 1). As shown elsewhere, these invasive spurs have been mistakenly described as " regenerating rete-pegs ", and we have consequently called them " pseudo-pegs ". Between the seventeenth and twenty-fifth to thirtieth post-operative days, with progressive fibrosis of the new connective tissue, the seemingly invasive epidermal pseudo-pegs become progressively thinner, and undergo internal keratinisation

(with or without neogenesis of sebaceous glands in man or of hair follicles in animals) and may become absorbed or separated from the surface epidermis, thus giving rise to epithelial pearl-like structures with an associated round cell infiltration or foreign body reaction. As a result of these processes, which are well advanced by the seventeenth to the twentieth post-operative days, the lower (dermal) surface of the new epithelium looses its serrations and becomes straight flat and " scar-like " (Fig. 2). Even after eight years the epithelium and connective tissue, in human Thiersch-graft donor sites, may easily be distinguished from the normal skin of the area studied. The stratum papillaris of the dermis apparently never regenerates ; collagen fibres, of both portions of the dermis, are arranged abnormally, and elastic fibres are rare, even eight years after the injury.

If, at the initial operation, *i.e.* removal of thin split skin grafts, grafts of trypsin-treated auto- or homo-dermis or (as shown by our more recent studies in human volunteers) even untreated composite homo-Thiersch grafts, two to three centimetres long by two centimetres wide, are applied, the course of healing is profoundly altered. In the case of trypsin-treated homo-dermal grafts, as previously described, epithelial regeneration is considerably stimulated, resulting in the covering of the graft itself by quite a thick scar-like layer of host epithelium. However, the graft is simultaneously undermined by a much thicker layer of host epithelium which grows excessively, resulting in the formation of numerous serrated processes ramifying in the new connective tissue in the wounded area (Fig. 3). This excessive epithelial activity is *not* confined to the graft site, but extends for 2–3 mm. around the homo-dermal grafts as indicated in Fig. 3. By the seventeenth post-operative day most of the graft has been shed in flakes by a complex process previously described (Gillman *et al.*, 1953). However, the epithelial activity, in and around the homo-dermal graft recipient area, is still maintained and has even increased to the proportions and in the manner depicted in Fig. 4 and 5. In these figures the epithelium can be seen to have given rise to numerous sheets or cords of cells penetrating deeply into the underlying connective tissues. At this stage the morphology of the epithelium is such as to resemble closely locally benign neoplasias of the basal-cell carcinoma (Fig. 4) or squamous epithelioma (Fig. 5) type. The local epidermal neoplasia-like growths were consistently encountered in sites treated with homo-dermal grafts, in *all* the ten human subjects from whom biopsies were taken between the fifteenth and eighteenth post-operative days. Biopsies of such homo-dermal graft recipient sites, taken after the twenty-third to twenty-fifth days, revealed complete resolution of the epidermal hyperplasia which was so prominent at an earlier stage.

However, in biopsies of these same sites, taken after the fortieth or even as late as the eightieth post-operative days (when the last biopsies were taken from our human cases) there were consistently encountered abnormal connective tissue and associated disturbances in epithelial growth. The abnormalities in the connective tissue, to which we particularly refer, are those portrayed here in Fig. 6 and 7. With ordinary haematoxylin and eosin staining the collagen fibres in these experimentally treated graft-donor site areas appeared coarser, unusually wavy, coiled, clumped or even granular, and were basophilic. These " abnormal collagen " fibres could always be sharply delineated by the use of any of the elastic tissue stains enumerated above. As a consequence they were initially taken as new but abnormal elastic fibres. More complete examination, by the use of many tinctorial and histochemical methods (some of which but not all are enumerated

above under " Material and Methods "), revealed that these " elastic fibres " differed from normal fine elastic fibres or from coarse elastic " membranes " encountered in the skin, and especially in blood vessels.

The main differences between " normal " collagen or elastic fibres and what we have called " elastotically degenerated collagen " have been detailed fully in other studies (Gillman *et al.*, 1954). In summary these differences are :

(1) The fibres are coarser and wavier than normal collagen bundles and may even be granular and clumped.

(2) They are distinguishable from collagen in that they are :

(*a*) Basophilic, with haematoxylin and other basic dyes.

(*b*) Heavily stained with recognised elastic stains such as Verhoeff's, Taenzer-Unna-Orcein and Weigert's methods.

(*c*) Chromophobic to the anilin blue in Mallory's triple connective-tissue stain, and almost so to the haematoxylin derivatives in Mallory's phosphotungstic acid haematoxylin.

(3) Although they stain with all recognised elastic stains, the elastotically degenerated fibres can be differentiated from " true " elastic fibres or membranes thus :

(*a*) By their morphology, although they approach in thickness normal elastic " membranes ".

(*b*) Whereas they stain with some of the elastic fibre stains, they fail to stain with other dyes which colour thick elastic membranes present in normal blood vessels and in dermis.

(*c*) On the other hand, they also stain with other dyes which do *not* colour " normal " elastic fibres or membranes (Gillman *et al.*, 1954).

It seemed unlikely, from the study of our material, that these long stretches or smaller clumps of elastotically degenerated fibres, encountered in donor sites treated with homo-dermis, could have been derived from the collagen fibres of the grafted homo-dermis itself. It is probable that this possibility can only be excluded by the use of radio-actively labelled homo-dermal grafts. However, we tend to the view that these elastotically degenerated fibres, which appear in healed Thiersch-graft donor sites treated with homo-dermal grafts, result from disturbances in normal collagen formation in the experimental site. We therefore refer to them as " elastotically degenerated collagen ". Proof of this view is, of course, still to be provided.

Our interest in these elastotically degenerated fibres lies in two main points :

Wherever such abnormal fibres were encountered, they were invariably associated with spurs of epithelium growing downwards either directly through, or more commonly around the edges of these fibre masses (Fig. 6 and 7). These latter epidermal spurs could become quite extensive and thick and were often seen to undermine completely the elastotically degenerated fibre masses. The latter thus often came to be completely, or almost completely, surrounded by a ring of epithelium. That portion of the surrounding epidermal ring immediately underlying the abnormal fibres, thickens and then seems to undergo internal keratinisation. Ultimately the elastotically degenerated fibre mass is sloughed off in flakes together with the original surface epithelium and that part of the deep epidermal bar which came to lie between the zone of internal keratinisation and the elastotically degenerated fibres. With recurrent sloughing, in small flakes, of the elastotically degenerated fibres encapsulated in epithelium, the *new* surface epithelium is

represented by that portion of the new epidermal bar which lies deep to the zone of internal keratinisation. This process of sloughing of clumps of elastotically degenerated fibres closely resembles the sloughing of a dried blood clot or "scab" which forms over an excised wound or an abrasion (Loeb, 1898; Gillman *et al.*, 1953).

EXPLANATION OF PLATES

FIG. 1.—Section of ten-day-old untreated healing Thiersch-graft donor site showing epidermal hyperplasia and "invasion" of *new* connective tissue by epidermal "pseudo-pegs". Weigert's elastic stain, haematoxylin-eosin. × 32.

FIG. 2.—Section of seventeen-day-old untreated Thiersch-graft donor site to show removal of serrations on lower surface of regenerated epidermis and the formation, in the new sub-epithelial connective tissues, of epithelial pearls and related foreign body reactions. Iron-haematoxylin. × 32.

FIG. 3.—Shows the well-marked epidermal hyperplasia and invasiveness occurring in relation to a small fragment of trypsin-treated homo-dermal graft (H.D.G.) which has become encapsulated in host epithelium at about the ninth post-operative day. Note that the epidermal hyperplasia is not localised only to the area immediately below the graft, but extends for some distance around the graft as partially shown at right of figure. Mallory's triple. × 12.

FIG. 4.—Showing the marked hyperplasia and invasiveness which is regularly encountered, at about the seventeenth post-operative day, in those parts of a Thiersch-graft donor site which had previously been treated with trypsin-treated homo-dermis. The degree of epithelial hyperactivity and invasiveness can be well appreciated by comparison of this figure with Fig. 2. Verhoeff's elastic stain. × 32.

FIG. 5.—Another example of the morphology and extent of the epidermal hyperplasia and invasiveness which supervenes in Thiersch-graft donor sites, seventeen days after the application thereto of trypsin-treated homo-dermal grafts. Such epidermal hyperplasias resemble, temporarily, local benign neoplasias which are subsequently eliminated. Weigert's elastic stain, Haematoxylin-eosin. × 32.

FIG. 6.—An example of localised areas of "elastotic degeneration" of collagen which are regularly encountered, some forty days after the healing of a Thiersch-graft donor site in areas which have been previously treated with trypsin-treated homo-dermis. Note also the tendency of epidermal spurs to invade the dermis at the edges and even through the substance of such elastotically degenerated plaques. Stained as for Fig. 5. × 43.

FIG. 7.—Another area of "elastotically degenerated" collagen appearing in a healed Thiersch-graft donor site some eighty days following the application thereto of trypsin-treated homo-dermis. The persistence of hyperplasia and invasions of the epidermis into such elastotically degenerated plaques and around the edges thereof is clearly evident. Stained as for Fig. 5. × 72.

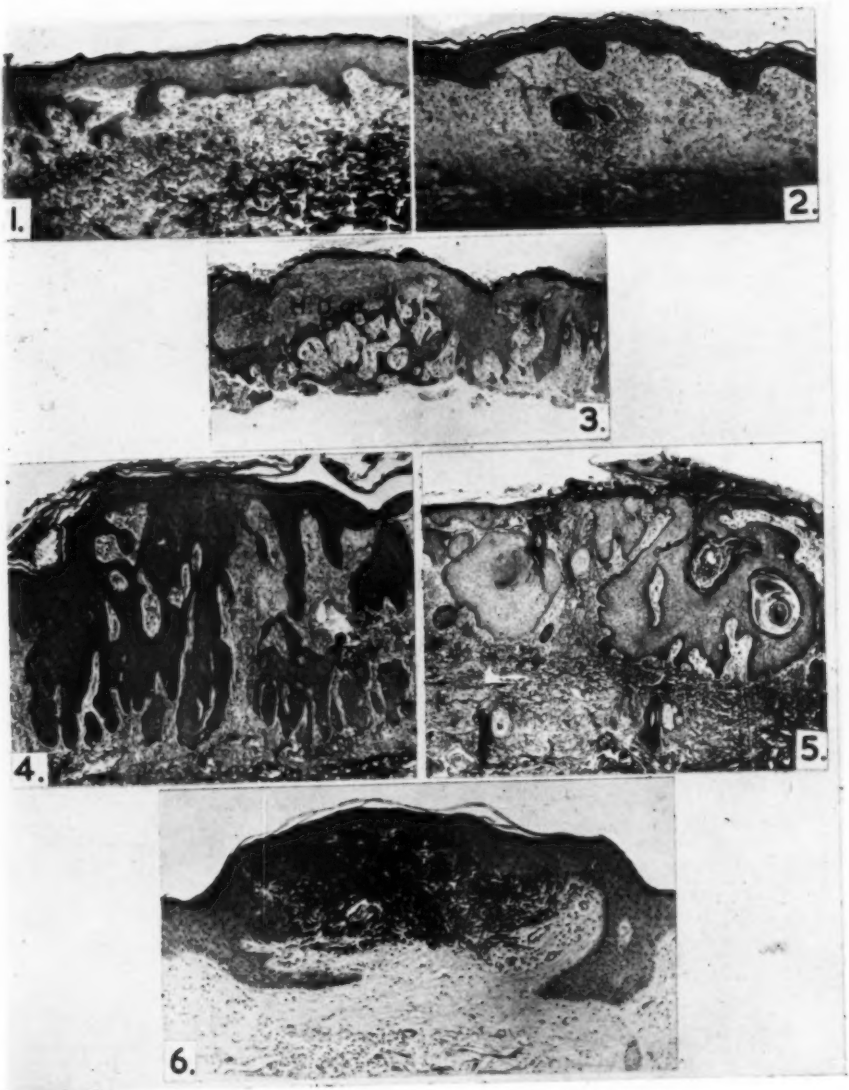
FIG. 8.—An area of epidermal hyperplasia and invasiveness associated with a known malignant tumour of the skin, for comparison with Fig. 4 and 5 above. The invasive epidermal mass at the left of this figure consists of malignant cells. Haematoxylin-eosin. × 43.

FIG. 9.—Another specimen of known epidermal carcinoma to show the invading epithelium, epithelial plaque-formation, early epithelial nest-formation and the strong round-cell infiltration evoked by this invasive epidermis. The hyperplasia of the epidermis and the formation of pseudo-rete pegs is also well demonstrated at the left of this figure. For comparison with Fig. 4 and 5. Haematoxylin-eosin. × 32.

FIG. 10.—Skin of dorsum of the hand from a case of chronic, pre-malignant ultraviolet irradiation dermatitis; showing the epidermal hyperplasia, invasion of short epidermal pseudo-pegs, and the associated large mass of elastotically degenerated collagen below. Between this elastotically degenerated collagen bed and the epidermis there has now appeared a zone of round-cell infiltration and new connective-tissue formation. For comparison with Fig. 6 and 7 above. Weigert's elastic stain, haematoxylin-eosin. × 32.

FIG. 11.—Showing similar mass of elastotically degenerated collagen lying in an extensive X-radiation scar. The atrophic nature of the overlying epithelium with the formation of small pseudo-peg invasions of the dermis are also clearly depicted. For comparison with Fig. 6, 7 and 10 above. Mallory's phospotungstic acid haematoxylin. × 32.

FIG. 12.—Showing a large mass of elastotically degenerated collagen in a twenty-seven-year-old burn-scar which has undergone malignant change following recent minor injury thereto. Once more the abnormal overlying epidermis and the formation of numerous new pseudo-pegs of invading epidermis are clearly depicted, together with true neoplasia (at left). Orcein-haematoxylin. × 72.



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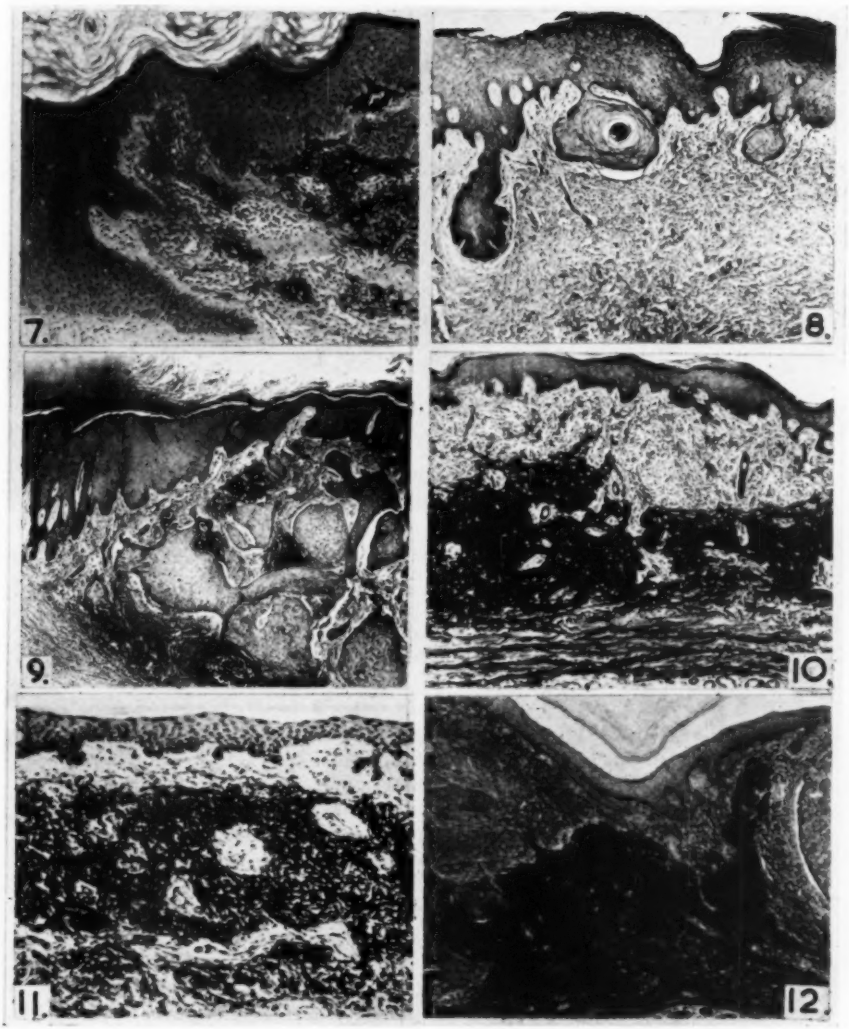

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Thus, these elastotically degenerated fibre masses once produced, even in "normal" skin, seem to evoke a definite invasive, albeit localised and benign "new growth" of epidermis which is evanescent but recurrent.

The second reason for interest in these elastotically degenerated collagen masses lies in the fact that we have yet to see a skin cancer in man which is *not* associated with similar elastotically degenerated collagen masses—of greater or lesser extent. Examples of such elastotically degenerated fibre masses, in pre-cancerous and frankly cancerous skin-lesions caused by chronic ultraviolet, X-ray or radium-irradiation, and also in association with a malignant old burn scar, are depicted in Fig. 10, 11 and 12. The morphology of the associated non-malignant and malignant epidermal hyperplasias and invasions of the dermis are portrayed in Fig. 8, 9 and 12, for comparison with the reactions produced experimentally in normal, non-irradiated human skin (Fig. 7 and 8).

The morphological, tinctorial and histochemical reactions of the experimentally induced elastotic degenerations are identical with those occurring in pre-cancerous or cancerous skin lesions in man. We have, however, also encountered similar elastotically degenerated collagen in the so-called elastica mimica of normal adult facial skin, in non-malignant old burn scars, and in degeneratory processes in the connective tissues, at least of arteries, and in the tunica propria of the gall-bladder.

DISCUSSION.

In the introductory section of this study, we have outlined several problems, relating to the histogenesis of skin cancers, which require solution if progress is to be made in understanding carcinogenesis. The findings which we have here presented permit the following broad general statements:

1. Epidermal invasions of the dermis are normal concomitants of the repair of acute and chronic skin injuries.

2. The supervention of such epidermal invasions seems to be related, directly or indirectly, to the activity of the connective tissue during certain stages of wound healing.

3. The invasive activity of the epidermis, during normal wound healing, seems to be brought under control by the activity of the connective tissue, the regeneration of which appears to be initiated by epithelial regeneration.

4. Disturbances of the connective tissue, in healing wounds, may *alone* bring about markedly increased, albeit an evanescent hyperplasia and invasiveness of the epithelium.

5. Disturbances of connective tissue, at least during the early stages of healing, may result in prolonged alterations in the connective tissue in the healed site, with associated epidermal activity, of major or a minor order, but persisting for at least eighty days.

6. Dermal changes, apparently identical in quality (but differing in extent, duration and no doubt also in mode of origin), are also consistently encountered in many degeneratory changes in the human skin associated with ageing, chronic radiation injuries or with pre-cancerous or frankly cancerous lesions.

In our experiments the collagen placed in the healing donor site (as a trypsin-treated homo-dermal graft) was abnormal in that, firstly, it was a homo-graft and, secondly, it had been previously subjected to digestion with trypsin. We now know that trypsin treatment is not essential and that the dermal fragments (from

untreated split-skin homografts) which remain after epidermal sloughing (Medawar, 1945) may, in time, evoke similar reactions, but to a lesser degree. In ageing skin, as in skin damaged acutely or chronically by various irradiations or by burning, similar dermal changes result from local or systemic alterations in the metabolism of the connective tissues in general, and of collagen in the repaired site in particular. Also, in the latter circumstances, elastotic degeneration of collagen is much more extensive and prolonged than in the conditions of our experiments reported above.

Whether *repeated* applications of abnormal connective tissue to traumatised non-irradiated young skin will ultimately permit the experimental production of epidermal neoplasms in animals or in man is still under investigation in this laboratory. We can state, at this stage, that composite healthy auto-grafts, and auto- or homo-grafts of epidermal or dermal tissue do profoundly alter the healing of standard experimentally inflicted excised wounds in rabbits. Either the epidermal or the dermal components may be significantly affected during healing by the application of various types of auto- and homografts. We are led to conclude, therefore, that the reactions of the epidermis in healing and probably also in carcinogenesis cannot be studied in isolation from the related connective tissue changes, since changes in the latter may profoundly influence the epidermis.

As for the second problem raised in the introduction, *i.e.* whether or not skin carcinoma invariably arises from preformed hair follicles, we feel that our studies of healing wounds and of carcinogenesis in man and in animals allow us to make certain suggestions.

Wolbach (1951) stated that neogenesis of hair follicles does not occur in the mouse, and, on the other hand, that neoplasms develop from : " ... hair follicles which have lost papillae rest cells ... the follicle deprived of its papilla is no longer the source of new follicle formations ... the explanation of focal origin of papillomas awaits better understanding of the conditions which result in the destruction of papilla cells."

To revert to our previously recorded findings on healing wounds—as stated above, epidermal " pseudo-pegs ", invading the connective tissue, consistently occur during wound healing in man and in more hairy animals, such as mice and rabbits. That neogenesis, at least of sebaceous glands and hair follicles, can occur in healed wounds or in scar epithelium now seems to be clearly established (*vide supra*). It may well be that the formation of invasive epithelial pseudo-pegs represents a reversion to an earlier ontogenetic behaviour characterising developing skin in the embryo (in man) or early post-natally in rodents.

Our findings in methylcholanthrene-treated skin of mice lead us to believe that the *original* hair follicles and sebaceous glands are usually *completely* destroyed by chronic treatment with this carcinogen. It seems that if new hair follicles and sebaceous glands form in sites severely injured by the carcinogen that they do so *de novo* from the equivalents of those invasive epidermal pseudo-pegs which we have described above, as occurring invariably during normal wound healing.

The reformation of hair demands at least two essentials :

(1) The presence of connective tissue competent to respond to epidermal down-growths, by forming new hair papillae. Even during embryological development both epidermal and dermal components are indispensable for normal hair formation.

(2) An epidermal invasion of the dermis capable, firstly, of acting as an evocator

of connective tissue to form a hair papilla and, secondly, of responding to the organizing actions of such dermal papillae.

There is ample evidence in the literature (Orr, 1939 ; Marchant and Orr, 1953 ; Ma, 1949) and from our own experimental studies that the dermis is profoundly altered by the application of carcinogens, and that these changes in the connective tissues precede epidermal neoplasia. It seems possible, therefore, that such dermal damage may result in an inability on behalf of the connective tissues to respond to the normally healing epidermal down-growths with subsequent failure to reform hair papillae. The latter seem to play an important rôle in organising the regenerated epidermal " Anlagen " into new hairs. Under these circumstances epidermal invasions occurring normally during healing would not be adequately controlled and may then, perhaps, become neoplastic.

In view of what we have said above, we submit, as a possibility, that carcinomas in man and in animals may result in any of those circumstances in which there are attempts at epidermal regeneration, with the associated normal invasions of the dermis by epidermal pseudo-pegs (or pseudo-epidermal hair " Anlagen " in animals), which *fail* to evoke in the dermis either (*a*) fibrosis with subsequent elimination of the pseudo-pegs, as in normal wound healing in man, or (*b*) hair-follicle formation with consequent organisation of the epidermal " invasions " into normal *new* hair follicles and/or sebaceous glands.

Most of the investigators who have considered alterations in the dermis as playing a rôle in the pathogenesis of skin carcinomas have usually stated that these dermal changes operate by interfering progressively with the nutrition of the epidermis (Wolbach, 1909 ; Vernoni, 1951). We have shown that alterations in the dermis may *alone* evoke marked hyperplasia and invasiveness of the epidermis. On the basis of these findings in man, as well as from our experimental work, we have suggested here a totally different manner by which damage to the *dermis* may contribute towards the histogenesis of skin cancers, namely, by failing firstly (in rodents) to form hair papillae and as a result to organise epidermal pseudo-hair " Anlagen " into hairs, or secondly (in man) to induce new sebaceous gland formation or to eliminate the epidermal pseudo-pegs which normally develop during healing.

That the connective tissues may play an important rôle in determining the fate of epidermal invasion of the dermis is shown by several experimental studies. In his classic studies of the reactions of auto- and homo-grafts in rabbits Medawar (1944) stated (p. 181) that in autografts : " Not merely the slope, but also the pattern of the mature hair follicles is controlled by the structure of the collagenous endoskeleton of the original graft. If the graft contained multiple nests of hair follicles, multiple nests are developed when the hairs are newly formed."

In the scar-like tissue surrounding auto-grafts, and over which epithelium generated from these grafts spreads, Medawar found that new hairs were *not* formed, and he states (p. 181) : " ... this is because the connective tissue endoskeleton is not of the right pattern, and not because the new epithelium, however ' immature ' it may appear to be, lacks any part of its normal capacity to manufacture new hairs."

Subsequently, in the same study, Medawar recorded the fate of the dermal pad, remaining after the epidermis of a homo-graft had sloughed. He stated (p. 186) : " Almost as soon as native epithelium reaches the foreign dermis, whether it approaches it from above or from below, hair-follicle primordia are laid

down. Evidently skin epithelium, however 'immature', has the power to manufacture hairs provided that it reaches a depot of collagen fibres of the right size and pattern of packing."

The possibility also exists that the failure of dermal tissue to promote new hair follicle formation may be due not only to unfavourable structural organisation of these connective tissues (as suggested by Medawar, 1944) but also to some *chemical* differences. More recently, Cairns and Saunders (1954) have shown that ectoderm-free *leg* mesoderm, implanted into the *wings* of chick embryos, influence the determination of the specific morphogenetic activities of the overlying ectodermal cells of the wing into scales, claws and feathers, characteristic of the leg. Teir, Voutilainen and Kiljunen (1954), on the other hand, claim to have induced neoplasia with methylcholanthrene in the normally resistant rat by simultaneously injecting embryonic skin suspensions. These studies provide indications of the importance of mesodermal derivatives in determining the direction of differentiation of foetal and even of adult surface ectoderm.

Both (or either) the state of the epidermis or of the underlying dermis may apparently be responsible for repeated epidermal pseudo-peg formation (*vide supra*), neogenesis of hair follicles and/or sebaceous glands or, finally, epidermal neoplasia. Viewed in the light of the above discussion, it would seem that the dermis plays at least a most important if not a dominant rôle in determining whether or not carcinoma will supervene in epidermis previously activated to regeneration in response to the effects of physical and/or chemical trauma.

SUMMARY.

1. During normal healing of Thiersch-graft donor sites in man epithelial "invasions" of the underlying connective tissues occur consistently. Such normal epidermal invasions can be markedly stimulated so that by the seventeenth post-operative day the epithelium, in healing donor sites, may for a while resemble a localised epidermal new growth. Such epidermal hyperplasia is followed, some weeks later, by abnormalities in the connective tissue in the healed donor site.

2. The epidermal hyperplasia, and the subsequent alteration in the related connective tissues mentioned above, can regularly be produced in human subjects by the application to fresh Thiersch-graft donor sites of trypsin-treated homodermal grafts. It is suggested that these changes are attributable to the trypsin-treated homo-grafts acting as "foreign" or abnormal collagen.

3. Alterations in the *connective tissues* of the healed donor sites were also found to occur some weeks after the initial operation. These changes are shown to be due to the development of abnormal fibres which stain with all known elastic tissue stains but which, on the basis of various criteria, are nevertheless considered to be "elastotically degenerated" collagen. These elastotically degenerated fibres are shown to be consistently related to intermittent, repeated and localised epidermal invasions of the dermis.

4. It is shown that similar elastotic degeneration of collagen is invariably present in the dermis in many degenerative skin conditions (due to chronic irradiations) which may and frequently do become pre-cancerous or frankly cancerous.

5. It is suggested that the elastotic degeneration of dermal collagen may play an important rôle in the pathogenesis of skin cancers. A possible explanation for the development of carcinomas in chronically traumatised skin is suggested, which maintains that carcinomas supervene when fibrosis or degeneration prevents the

dermal tissues either from eliminating or, alternately, from inducing hair formation in those epidermal pseudo-pegs which invade the dermis during normal repair. This suggestion allows for some understanding and further experimental testing of the view that epidermal carcinogenesis is directly related to hair regeneration in man and in animals.

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THE PRODUCTION OF MAMMARY CANCER IN RATS USING OESTROGENS.

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THE primary objects of this investigation were : (*a*) to confirm the observation made by previous workers, notably Noble, McEuen and Collip (1940), Geschickter and Byrnes (1942), Nelson (1944) and Dunning, Curtis and Segaloff (1947), that mammary cancer can be induced in rats by prolonged stimulation with oestrogenic substances ; and (*b*) to determine whether there is any variation in the response of different strains of rats to the carcinogenic action of these oestrogens on their mammary glands.

In general, it may be said that the results obtained amply confirm the previous reports with regard to the production of malignant tumours in rats by oestrogens, but that these results appear to vary with the strain of rat employed.

Certain other facts were noted in the course of the experiment and these will be discussed in due course.

MATERIALS AND METHODS.

Rats of three different strains were used in these experiments :

(*a*) Albino rats obtained from the research laboratories of the Royal Cancer Hospital, London. These were the descendants of animals which had originally been obtained from a commercial source, and had been bred in a random fashion for a good number of years ; brother-to-sister mating had never been attempted. An exceedingly low proportion, one in many thousands, develop simple adenoma of the breast, sometimes multiple, but a malignant tumour of the breast has never been seen. For the purposes of this investigation, six male and six female rats, all young adults, were obtained and the animals used in these experiments were bred from them by random mating.

I am indebted to Professor A. Haddow of the Chester Beatty Research Institute for the supply of these animals and for the details of their previous history.

(*b*) Albino rats obtained from the Glaxo laboratories, known as WAG rats. They were originally derived from the Wistar albino strain and had been maintained by brother-to-sister mating from the time of their introduction to the Glaxo laboratories ; when the animals used in these experiments were first obtained about 50 generations had been so bred.

Spontaneous mammary cancer is extremely rare in this strain, though exact figures are unfortunately not available. For the purposes of this investigation, 12 male and 12 female young rats (5 weeks old) were originally obtained. Twelve of these (6 male and 6 female) were kept for breeding purposes, brother-to-sister mating being continued, and from these 6 pairs were bred the animals designated

WAG rats in these experiments. The remaining 6 pairs were used for experimental purposes.

(*c*) Black and white (hooded or piebald) rats maintained in the laboratories of the Medical School, Newcastle upon Tyne, formed the third group. They are descendants of rats which had been obtained from a colony at the M.R.C. laboratories at Mill Hill. This colony was derived from black and white rats which had been obtained from the laboratories of Imperial Chemical Industries some years previously, and they in their turn were descended from animals obtained from the Lister Institute which were reputed to be derived originally from hooded rats of the Wistar Institute. In the M.R.C. laboratories they had been kept for several years as a closed colony, but breeding was by random mating, no true inbreeding having been attempted. In Newcastle this method of breeding was continued and young animals were obtained from these matings as required for the experiments. No accurate figures as to the occurrence of spontaneous mammary tumours in this strain are available, but in the I.C.I. laboratories it had been found that a good many of the older females developed benign mammary tumours (fibro-adenomata), and occasionally a mammary carcinoma was noted. For the duration of the experiments recorded here, a period of about two and a half years, all the adult animals in the colony were examined periodically for mammary tumours; out of several hundred animals, five such tumours, which will be described later, were found.

It will thus be evident that, of the three strains, only one (Glaxo WAG rats) can be described as a pure strain, comparable to similar strains of mice. Of the other two, the Cancer Hospital stock may be regarded as semi-inbred, while the hooded rats were probably the least inbred of the three groups.

The rats were fed throughout the experiments on a diet of rat cake of the following composition (as obtained from the makers, The North-Eastern Agricultural Co-operative Society, Abderdeen):

Dried Milk	7·0
Oil	4·4
Albumnoids	20·3
Fibre	4·3
Carbohydrates	51·6
Salt	0·9
$CaCO_3$	3·3
Moisture to	100

This diet was supplemented by green vegetables and an unlimited supply of clean drinking-water was always available.

The oestrogens used were (*a*) oestradiol (Organon), (*b*) oestradiol dipropionate (Ciba) and (*c*) stilboestrol (Organon). These were implanted as pellets weighing 5–6 mg., which were made from the crystalline substances by a specially designed hand compression machine. Every rat was implanted with two such pellets of the same substance, the initial implant being made at the age of 4 weeks, into the muscles of the lumbar region on one side through a small skin incision, which was then sutured; the second implant was made 1–3 months later into the lumbar muscles of the opposite side in a similar fashion.

The animals were examined for mammary tumours at regular intervals, and at

death a complete post-mortem examination was carried out, a search being made for gross tumours of all organs. Portions of the mammary glands (where these had not atrophied completely, as happened in a considerable number of animals), thyroid, adrenals, sex organs, pituitary and any other tissues of interest were preserved for microscopic section. A search was also made at the sites of implantation of the pellets, but in the great majority of the animals these had been absorbed completely by the time death occurred; in the few cases where they were still present they were less than half their original weight. In no instance was there any evidence of local tumour production.

RESULTS.

The total number of experimental animals (female) used was 140. Table I summarises the numbers of each type of rat used, the numbers implanted respectively with oestradiol, or its dipropionate derivative, and stilboestrol, and the number of malignant mammary tumours obtained. These results are shown graphically in Fig. 1–6.

TABLE I.—*Mammary Cancers Developing in Rats Treated with Oestrogens.*

Strain of rat.	Numbers employed (females only).	Number implanted with: Oestradiol or oestradiol dipropionate.	Number implanted with: Stilboestrol.	Number with Mammary cancer. Oestradiol group.	Number with Mammary cancer. Stilboestrol group.	Total. Number.	Total. Per cent.
WAG	46	29	17	10	0	10	22
Cancer Hospital	61	38	23	2	1	3	5
Newcastle hooded	33	25	8	6	2	8	24
Total	140	92	48	18	3	21	15

General Observations.

The animals implanted with oestradiol or its dipropionate showed a definite retardation of growth as compared with unimplanted controls. This retardation in growth rate began as soon as they had been implanted and was progressive, the average weight at death being about 110 g. as compared with an average weight of 225 g. for the unimplanted controls. On the other hand, this retardation in growth and difference in weight was not nearly so marked among those animals implanted with stilboestrol, in which group the average weight at death was 210 g. (these figures refer to females only) (Table II).

TABLE II.—*Retardation of Growth of Female Rats Implanted with Oestrogens.*

Rats implanted with:	Numbers with pituitary tumours.	Average body-weight at death. g.	Average pituitary weight. mg.	Range of pituitary weight. mg.
Oestradiol or oestradiol dipropionate	69 (out of 92)	110	320	29–606
Stilboestrol	19 (out of 48)	210	34	13·5–90
Controls	0	225	14·5	9–24

In addition to this retardation in weight the oestradiol implanted animals showed loss of activity and partial loss of hair. They also frequently showed ataxic symptoms, which were associated with the increased size of the pituitary. The stilboestrol implanted animals on the other hand showed little or no loss of activity, no loss of hair and only occasionally mild ataxic symptoms.

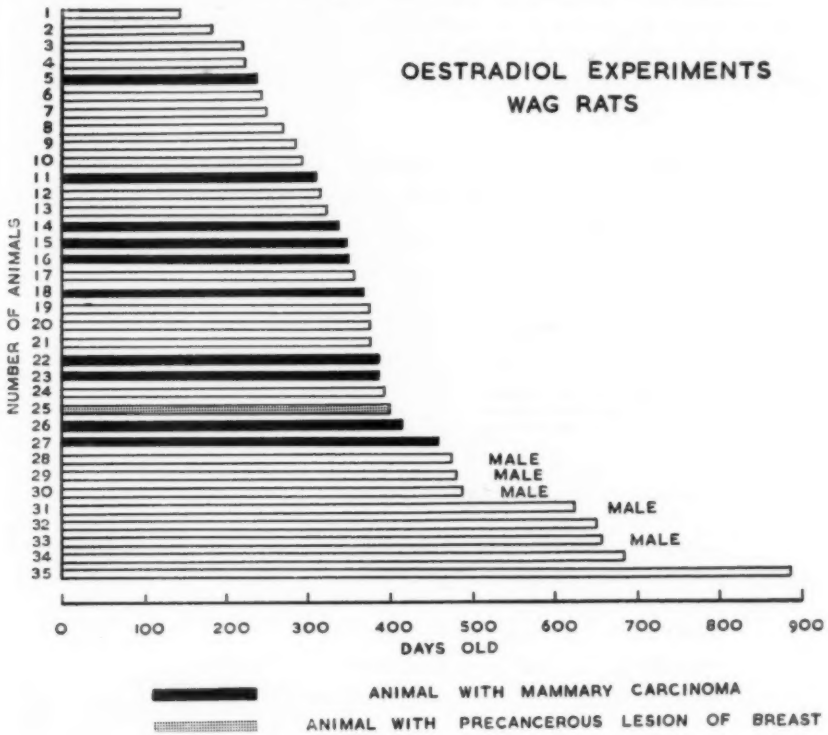


Fig.1

Histology.

It was found that the changes produced by (*a*) the naturally occurring oestrogen oestradiol, and its dipropionate derivative, and (*b*) the synthetic oestrogen di-ethyl-stilboestrol, were sufficiently distinct as to allow of their being discussed separately.

(a) *Oestradiol and oestradiol dipropionate.*

No qualitative difference in the effects produced by these two substances on the mammary tissue of rats was noted. The numbers of animals of the different strains used were not large enough to allow of any conclusions being drawn as to any quantitative difference in their effects.

In a considerable number of the experimental animals the main effect on the breast tissue was one of atrophy, the mammary tissue being scarcely visible to the naked eye and, microscopically, consisting of a few acini and ducts in a very scanty stroma. The typical reaction, however, was one of cyst formation, the microscopic appearance being somewhat analogous to that of human chronic cystic mastopathy. There was, however, one major difference between the experimental condition in the rat and the naturally occurring human condition, and that lay in the degree of stromal reaction present. In the rat this was minimal, though there was some variation between different animals. The typical picture

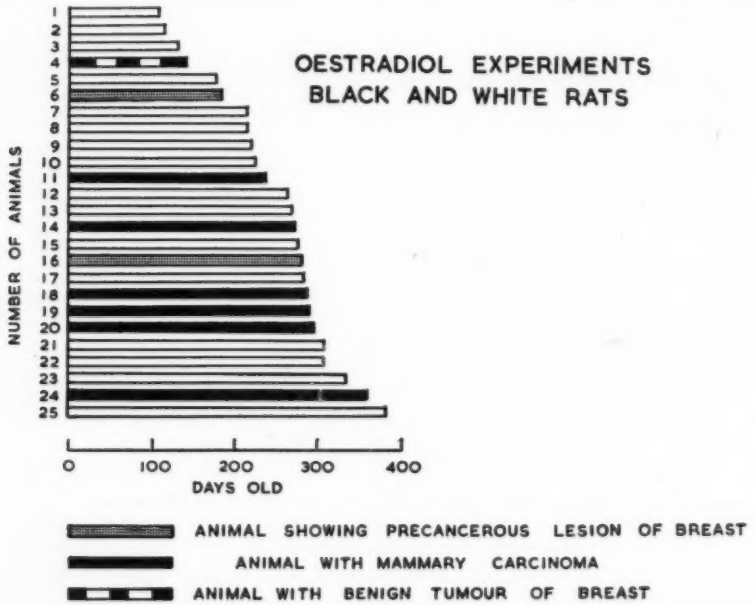


Fig. 2

was of numerous cysts of varying size, a few being sufficiently large to be visible to the naked eye, lined by cubical or flattened epithelium and filled with an eosinophilic structureless material in which desquamated cells or cell nuclei were sometimes visible. In some cases epithelial proliferation of the cells lining the cyst walls was seen and in a few this took the form of papillary ingrowths. In the majority of cases this proliferation was benign, but occasionally the cellular appearance suggested a pre-malignant change (Fig. 7).

Malignant change.—In 18 animals with malignant mammary tumours 10 had only one mamma involved, while in the remainder there were multiple tumours.

The histological picture varied from animal to animal, from one tumour to another in the same animal and in different parts of the same tumour but, in general, several different types could be distinguished. These were (*a*) adenocarcinoma, which was not common; (*b*) papillary carcinoma (Fig. 8); and (*c*)

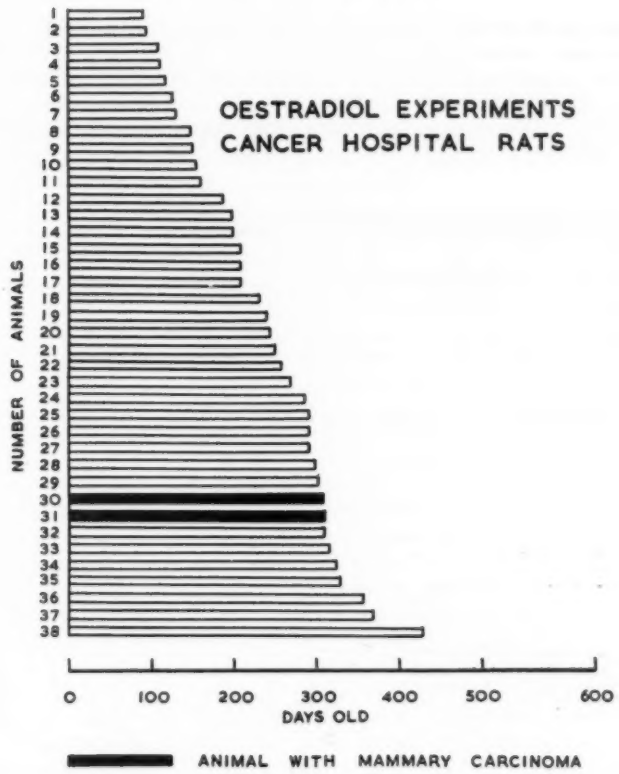

OESTRADIOL EXPERIMENTS
CANCER HOSPITAL RATS
NUMBER OF ANIMALS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
0
100
200
300
400
500
600
DAYS OLD
ANIMAL WITH MAMMARY CARCINOMA

Fig. 3

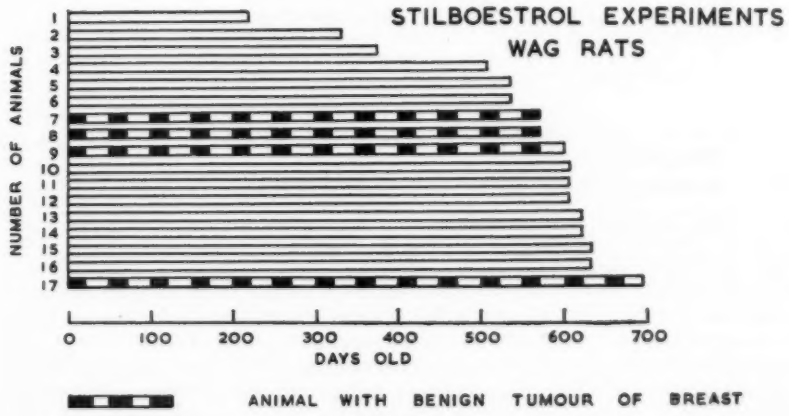

STILBOESTROL EXPERIMENTS
WAG RATS
NUMBER OF ANIMALS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
0
100
200
300
400
500
600
700
DAYS OLD
ANIMAL WITH BENIGN TUMOUR OF BREAST

Fig. 4

anaplastic carcinoma (carcinoma simplex), in which the cells were spheroidal in type and arranged in masses. A feature of many of the tumours was the occurrence of squamous metaplasia, which in some cases was very well marked, while

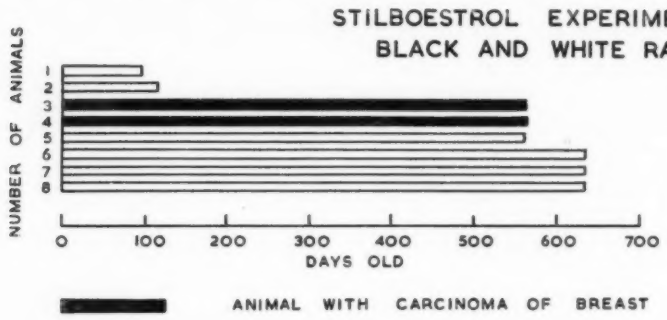


Fig. 5

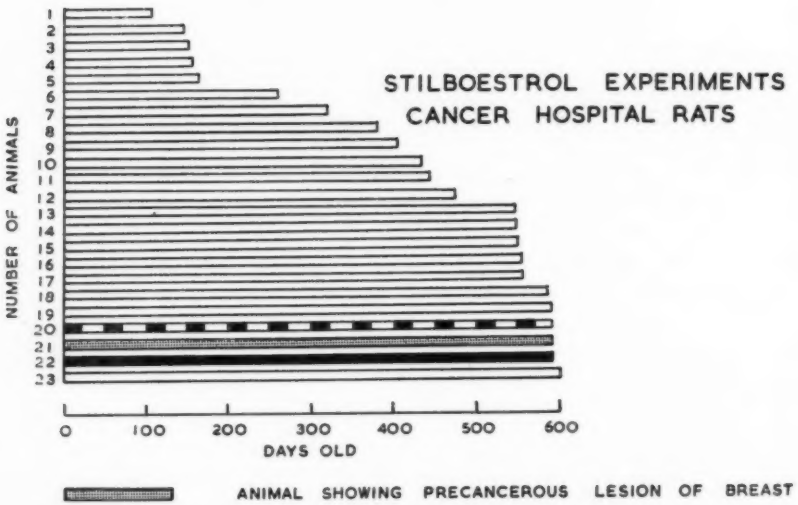


Fig. 6

in others it was seen only in one small area of the section (Fig. 9). It was never seen in any of the control mammae examined, nor in those of experimental animals which showed only atrophy or cystic mastopathy without malignant change.

The amount of fibrous tissue stroma in the tumours varied. In some it was minimal, but in the majority there was quite a well-defined stroma ; in only two

instances, however, was there anything analogous to the typical scirrhous carcinoma of the human breast, and then only in a portion of the tumour.

Involvement of the entire breast in the malignant process was not uncommon, while in other instances the tumour was confined to a portion of the mamma, the remainder showing the cystic appearance already described. Invasion of the muscular layer underlying the breast, by malignant cells, occurred in some cases, but in no instance were distant metastases noted.

(b) *Diethyl-stilboestrol.*

The effects of this substance on the mammary glands differed noticeably from that of the naturally occurring oestrogen and its derivative. (1) There was much less tendency to atrophy of the glands which, in the majority of the experimental animals, were well formed. (2) Cystic dilatation of the ducts and acini was fairly frequent but, in addition, there was frequently a marked but perfectly regular proliferation of the acini—adenosis. In some this resulted in well-defined systems of lobules, resembling the appearance of pre-lactational breasts in the controls, while in others the arrangement was more haphazard, though still quite benign. (3) There was usually a greater amount of fibrous tissue stroma in these glands, though it varied considerably, and in some there was very little. (4) Simple tumours, which occurred only once in the first group, were found in several animals. These were : (*a*) true adenomata each consisting of a mass of regularly formed acini, whose lining cells were perfectly regular, the whole being well encapsuled in the breast substance ; and (*b*) fibroadenomata in which the histological picture was that of an abundant fibrous tissue proliferation in which acini were embedded and compressed, and resembling very markedly the corresponding benign human tumour. (5) Only three malignant tumours occurred. Two of these were adenocarcinomas (Fig. 10), the third being a carcinoma simplex. Nothing resembling the varied histological structure of the first group was seen in them and squamous metaplasia did not occur.

Mammary tumours in control rats.

No mammary tumours were found in the animals of the control groups of WAG and Cancer Hospital rats. These groups were not large, but were approximately equal in numbers to, and of considerably longer average lifespan than, the animals in the experimental groups.

As the data on the occurrence of spontaneous tumours in the Black and White (hooded) strain were scanty several hundreds of these animals in the colony were kept under observation as controls during the period of the experiment. Among these five breast tumours were noted, as follows :

(1) A male, 409 days old, had two tumours, one in each flank in the lumbar region, 3 cm. and 2·5 cm. in diameter respectively. The histological appearance of each was identical, both being invasive, pleomorphic spindle-cell sarcomata with no evidence of epithelial elements (Fig. 11). Their positions suggested a mammary origin, but the histological appearance did not tend to support this.

(2) Two female rats, both 458 days old (born on the same day and probably, though not certainly, litter mates), each had a tumour in the left inguinal mamma, the one measuring 3·5 cm. and the other 5 cm. in diameter. Both were fibro-

adenomata in which the epithelial elements were predominant, but whereas in the one the stroma was scanty and surrounded the lobules into which the tumour was divided, in the other it was more abundant, oedematous in appearance and penetrated into and subdivided the lobules.

(3) A female rat, 606 days old, had a tumour in the right axillary breast measuring 1·5 cm. in diameter. Histologically, it was a fibroadenoma with a marked preponderance of fibrous tissue stroma, the epithelial elements being scanty and compressed (Fig. 12).

Effects on the pituitary gland.

A constant feature of the findings in the experimental animals was enlargement of the pituitary, though the degree of enlargement varied widely in different animals. Table II shows the average weight of the pituitary in the groups treated with oestradiol (or its dipropionate derivative) and stilboestrol, compared with the average weight of the gland in normal controls. The average body weights at death in the different groups are also shown.

In estimating the weight of the pituitary the gland was first removed entire and fixed in 10 per cent formol-saline. It was then removed from the fixative, all excess moisture removed with absorbent paper, and thereafter it was weighed on a sensitive chemical balance.

It was noticeable that the larger glands were found in animals which showed the greatest degree of weight loss and ataxia and that generally speaking the enlargement was considerably greater in the oestradiol treated groups than in the stilboestrol treated group.

In all instances the gland was removed from its surroundings without difficulty and nothing was seen to suggest, to the naked eye, that invasion of the adjacent structures had occurred.

Microscopy.—The microscopic findings were uniform, with some minor and one major difference. The proliferating cells were the chromophobes, and while, in some of the small glands, numbers of eosinophil cells and fewer basophil cells were seen, in the larger glands these were absent. These chromophobe cells were arranged in columns or masses in close association with thin-walled blood channels and were of uniform size and shape. The nuclei were as a rule well formed, though pyknosis was fairly frequent. No undue nuclear activity was noted and nothing was seen to suggest the occurrence of malignant change. The blood channels were numerous and thin-walled and varied in size, the largest channels being found in the larger glands. Not infrequently areas of haemorrhage (some quite large) had occurred into the substance of the glands.

One quite marked difference was noted between the oestradiol-treated and the stilboestrol-treated groups. Whereas in the former, an occasional gland showed a cyst-like space containing a structureless eosinophilic colloid material; this was of frequent occurrence in the stilboestrol-treated group. Commonly, there were several such colloid-filled spaces scattered through the substance of the gland in the latter group of animals, though, as noted above, they were on the average considerably smaller than in the oestradiol group. This may indicate that the pituitary cells were still functioning with some degree of normality in the stilboestrol group, whereas their secretory function was grossly impaired (if not entirely lost) in the oestradiol treated animals.

Other tumours in experimental animals.

Benign tumours.—A fairly frequent finding was an enlargement of one or both ovaries, which in some cases was very marked, the tumours measuring up to 2·5–4 cm. in diameter. This enlargement was due to simple cyst formation, a thin wall containing fluid contents or semi-solid or inspissated caseous material. Microscopically the wall was composed of connective tissue of varying thickness with an epithelial lining; in the larger cysts the latter was not always present. It was noticeable that whereas in the animals implanted with oestradiol or its dipropionate the content was almost always composed of an inspissated caseous material; in those implanted with stilboestrol it was generally fluid.

Occasionally similar tumours were found in the control groups, but they never reached the size seen in many of the experimental animals.

Malignant tumours.—Four extra-mammary malignant growths were observed in the experimental animals. Two of these were ovarian (Fig. 13), and 2 were uterine in origin (Fig. 14). They all occurred in animals implanted with stilboestrol, 3 of them in Cancer Hospital rats and the fourth (which was uterine) in a hooded rat. Their ages at death varied from 381 days to 602 days.

Microscopically all the tumours were squamous epitheliomata showing widespread local invasion, but no definite metastases were found.

No such malignant tumours were found in any of the control animals.

DISCUSSION.

Production of mammary tumours in rats.

The results of these experiments in general corroborate the observations of others on the occurrence of mammary cancer in rats following the prolonged stimulation of these animals by oestrogenic substances, and help to clarify the discrepancies in the incidence of such tumours in the various recorded experiments. In spite of much experimental work on the effects of oestrogens on rats (Hartwell (1951) has listed some 130 papers on the subject between 1930 and 1947), both male and female, intact and castrated, only a relatively small number of workers have obtained mammary tumours in these animals.

A study of the methods employed in these experiments however quickly reveals the reasons for this divergence in results, and helps to indicate the experimental conditions requiring to be employed in order to obtain such tumours.

These conditions can be summarized under two main headings: (*a*) the experimental animals and (*b*) the oestrogenic stimulation. With regard to the former the following points are of importance.

(a) *Numbers.*—In a species such as the rat, in which naturally-occurring mammary tumours are very uncommon, one may reasonably assume that the incidence of experimental tumours, using any given strain, may not be high and, accordingly, a sufficient number of animals must be included in the experimental series to allow for this. Too many of the experiments hitherto recorded have made use of far too small numbers of animals, and it is noteworthy that, in those experiments in which tumours have been recorded, the numbers of animals used have been much more adequate.

(b) *Sex.*—For the same reason female rats should be used first in any exploration of the experimental production of mammary cancer. Once the fact has been established that such tumours can be obtained in any given strain, then the

investigation of their production in males and in castrated or otherwise abnormal animals can be undertaken.

(c) *Age.*—The demonstration of the milk-agent in mammary cancer of mice and the fact that it may lose its effectiveness as the age of the animal increases before being infected (Andervont, 1945), shows that the process which determines the development of these tumours in mice is effective from a very early age. One may assume as a working hypothesis that a similar process, if present in another species, may work in much the same manner, and therefore the younger the animals are when the experimental conditions are begun the better.

It is noteworthy that the workers who have successfully produced mammary tumours in rats have all used young animals, the ages varying from 5–7 days (Noble, McEuen and Collip, 1940) to 3–4 months (Dunning, Curtis and Segaloff, 1947) though Geschickter and Byrnes (1942) were also able to produce tumours in animals up to 9 months old at the beginning of the experiment. In such animals, however, large doses of a particularly potent oestrogen were required. An exception to this generalization is the work of Eisen (1942) who was able to obtain only two mammary tumours among 134 rats which were 3–5 weeks old at the beginning of the experiments ; a possible reason for this low incidence is discussed below.

In the experiments recorded in this paper the age of the animals at the beginning of the experiments was standardized at 28 days, thus eliminating any discrepancy due to difference in age.

Strain.—In mice the existence of strains with an incidence of mammary cancer varying from zero to a high percentage has long been known and the demonstration of the presence or absence of the milk agent in such strains indicates the reason for the varying incidence. In no other species have such nearly homozygous strains been developed, but in quite a number of laboratories both in this country and the United States groups of rats have been inbred over a considerable number of years, to such an extent that though the breeding methods used have not been of the standard used in mice, yet it may be reasonably assumed that the animals within each group are closely related genetically and that one group differs from another at least as much as do the different races of mankind. Such an assumption may help to explain the varying incidence of mammary tumours obtained by different workers and emphasizes the necessity for an investigation of the incidence of such tumours in different strains of rats under strictly comparable experimental conditions.

EXPLANATION OF PLATES.

FIG. 7.—Section of breast from oestradiol treated rat showing precancerous proliferation within the ducts. × 128.

FIG. 8.—Section of breast from oestradiol treated rat showing papillary carcinoma. × 128.

FIG. 9.—Breast from oestradiol treated rat showing spheroidal cell carcinoma with squamous metaplasia. × 128.

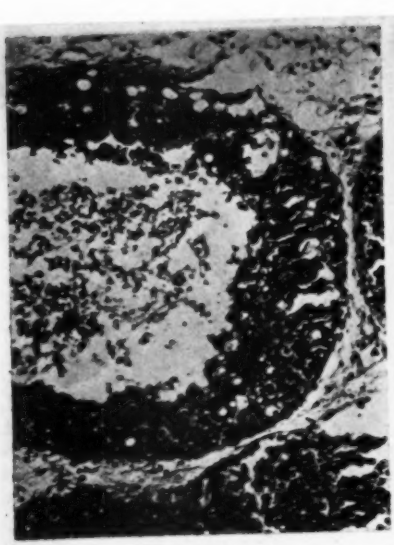
FIG. 10.—Breast from stilboestrol treated rat showing adenocarcinoma. × 128.

FIG. 11.—Section of breast tumour from control rate (hooded). Pleomorphic celled sarcoma. × 128.

FIG. 12.—Section of fibro-adenomatous tumour from breast of control rat. × 128.

FIG. 13.—Section showing carcinoma of the ovary from a stilboestrol treated rate. ×128.

FIG. 14.—Section showing carcinoma of uterus in a stilboestrol treated rat. × 128.



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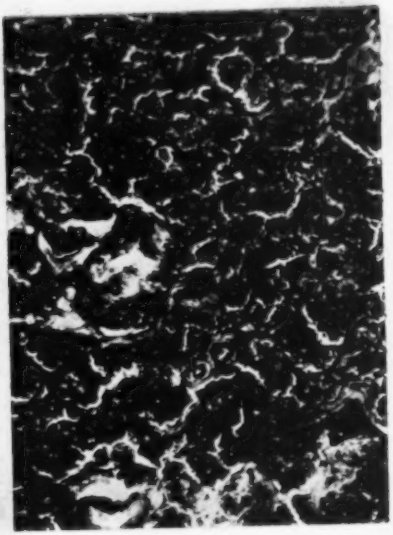

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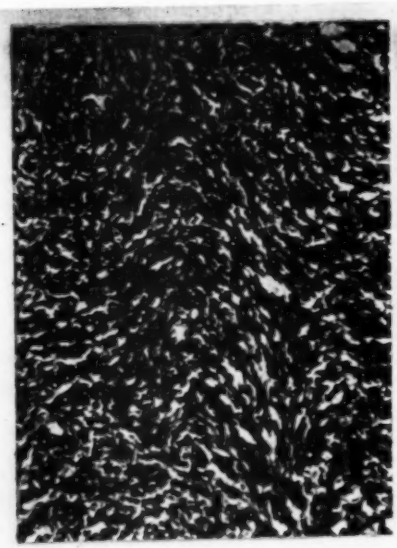

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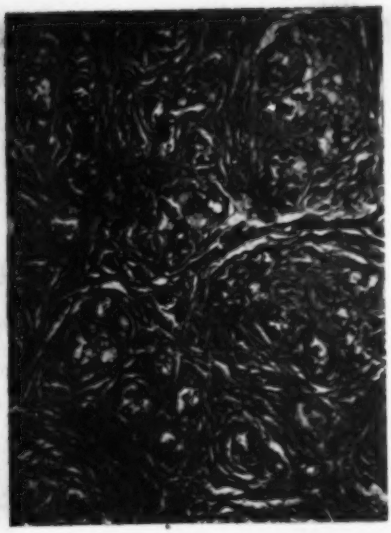

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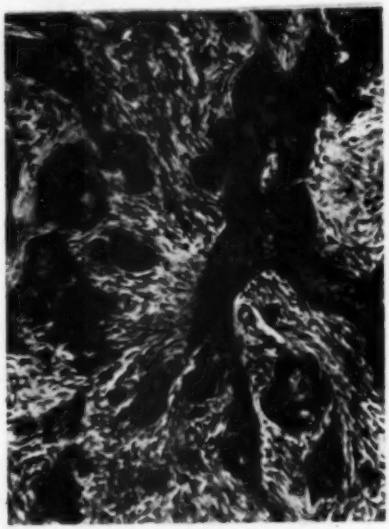

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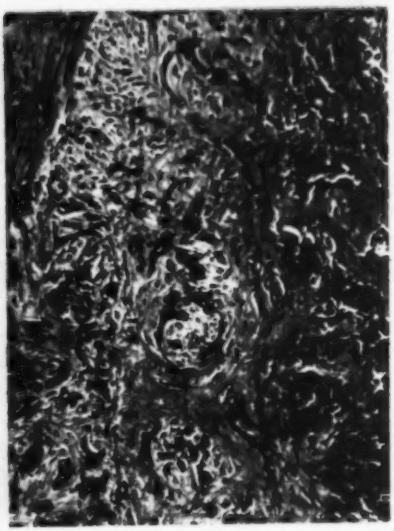

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The results obtained in the experimental part of this paper indicate that there is indeed a considerable difference in susceptibility among different strains of rats, and from these results and those of others one can divide them provisionally into high, medium and low cancer strains. Table III shows the strains, so far tested, assigned to each group.

TABLE III.—*Showing Susceptibility to the Development of Mammary Cancer by Oestrogen Stimulation among Strains of Rats so far Tested.*

High cancer strains.	Per cent.	Medium cancer trains.	Per cent.	Low cancer strains.	Per cent.
Hooded rats (Unknown ancestry) (Noble *et al.*, 1940)	55	Inbred albino rats (Geschickter and Byrnes, 1942)	36·4	Inbred Sherman rats (Eisen, 1942)	1·5
Long-Evans hooded rats (Nelson, 1944)	66	A × C line 9935 (Dunning *et al.*, 1947)	33	Copenhagen line 2331 (Dunning *et al.*, 1947)	0
		Newcastle hooded rats (MacKenzie)	24	Fischer line 344 (Dunning *et al.*, 1947)	6·3
		WAG rats (MacKenzie)	21·7	Cancer Hospital rats (MacKenzie)	5

It should be stressed however that these figures are not strictly comparable, in that (*a*) the ages of the animals at the beginning of the various experiments varied and (*b*) the oestrogen used, its dosage and method of administration also varied. Further work requires to be done to clarify and extend this observation.

Oestrogenic substances employed.

A considerable number of oestrogens have been used by various workers and Table IV summarises the results obtained. It should be emphasized, however that the experimental conditions under which they were used differed widely, and the fact that no tumours were obtained with any particular substance does not necessarily imply that it does not possess tumour-evoking properties. Until they have all been tested under identical conditions of dosage and time, using a strain of rats of known susceptibility, no definite conclusions can be drawn. In the present work tumours were obtained with both oestradiol and its dipropionate derivative, but the experimental conditions used were not such as to warrant any conclusions being drawn as to their relative effectiveness. Stilboestrol, on the other hand, while evoking the usual cystadenosis and occasional benign tumour, produced only 3 malignant tumours among 48 animals in these experiments. In view of the positive results obtained by others (Geschickter and Byrnes, 1942 ; Nelson, 1944 ; Dunning, Curtis and Segaloff, 1947) there must be some reason for this, and a review of the methods used by these workers suggests that this may be a matter of dosage. The amount used in the present experiments was a total of 10 mg. implanted intramuscularly as 2 pellets, whereas both Nelson and Dunning and his co-workers used a larger quantity. The latter workers, recognising that stilboestrol alone is absorbed fairly rapidly, incorporated it in pellets mixed with cholesterol in the proportion of 1 to 4. This greatly slowed the rate of absorption of the stilboestrol, and only after they had done so were they able to obtain breast tumours in their experimental animals. Geschickter and Byrnes (1942), on the other hand, were regularly able to obtain breast tumours with no more than 10 mg. of stilboestrol implanted subcutaneously as pellets. However,

the general appearance of the stilboestrol implanted animals in the present series (particularly their weight and the relatively small increase in size of their pituitaries) strongly suggested that the dosage was inadequate, and further work utilizing in particular the technique of Dunning, Curtis and Segaloff (1947), is required to clarify this point.

TABLE IV.—*List of Oestrogens Employed Experimentally in Rats.*
(Summarized from Hartwell, 1951)

Oestrogen.	Breast tumours.
Oestradiol	Tumours obtained (Geschickter and Byrnes, 1942: MacKenzie).
„ benzoate	Tumours obtained (Mark and Biskind, 1941; Geschickter and Byrnes, 1942).
„ dipalmitate	No tumours.
„ dipropionate	Tumours obtained (Eisen, 1942; Geschickter and Byrnes, 1942; MacKenzie).
Oestradiol-17-caprylate	No tumours.
Oestradiol di-n-butyrate	„ „
„ di-caprate	„ „
„ di-n-hexanoate	„ „
„ di-isobutyrate	„ „
„ di-n-octanoate	„ „
Oestradiol-3-benzoate-17-acetate	„ „
Oestradiol-3-benzoate-17-butyrate	„ „
Oestradiol-3-benzoate-17-propionate	„ „
Oestradiol-3-benzoate-17n-valerate	„ „
Oestradiol-3n-butyrate-17-benzoate	„ „
Oestriol	„ „
Oestrone	Tumours obtained (McEuen, 1938; Geschickter, 1939*a*, 1939*b*; Geschickter and Byrnes, 1942; Noble *et* al., 1940; Nelson, 1944).
Oestrone benzoate	Tumours obtained (Chamorro, 1943).
Oestrone-n-hexanoate	No tumours.
Oestrone-n-octanoate	No tumours.
Stilboestrol (4,4′-dihydroxy-αβ-diethyl-stilbene)	Tumours obtained (Geschickter and Byrnes, 1942; Nelson, 1944; Dunning *et al.*, 1947; MacKenzie).
Stilboestrol mono-methyl ether „ di-methyl ether	Tumours obtained (Geschickter and Byrnes, 1942).

The substances which have so far been successfully used in the production of breast tumours are oestradiol and its benzoate and dipropionate derivatives—oestrone and oestrone benzoate—and stilboestrol and its mono- and di-methyl derivatives. Of these Geschickter and Byrnes (1942) regarded oestradiol benzoate as the most effective.

Dosage.—Table V gives the dosages used in the various successful experiments, and it will be seen that they vary considerably. However, from a study of the various papers it would seem probable that a minimum of 10 mg. in pellet form of an active oestrogen is required to produce mammary cancer in rats and this agrees substantially with the conclusions reached by Geschickter and Byrnes (1942). In the case of a substance such as stilboestrol, which is rapidly absorbed, some agent like cholesterol, which retards absorption, should be mixed with it before the pellets are made.

Method of Administration.—Two methods have been used: (*a*) subcutaneous injection of the pure substance dissolved in oil and (*b*) subcutaneous or intramuscular implanation of pellets of the pure substance or of the pure substance mixed with cholesterol. Both of these methods have been successful in producing

TABLE V.—*Dosage of Oestrogens Used by Various Workers to Produce Tumours in Rats.*

Author.	Strain of rat.	Oestrogens employed.	Method of administration and age at beginning of experiment.	Number with mammary cancer.	Age at first appearance of cancer (days).
McEuen (1938)	Hooded rats of unknown ancestry (ovariectomized)	Oestrone in corn oil 30 μg. daily	Subcutaneously; 106 days	1/22	624
Noble *et al.* (1940)	Same strain (intact)	Oestrone 4–11 mg. pellets	Subcutaneous implantation; 5–7 days	28/49	226
Geschickter and Byrnes (1942)	Inbred albino	Oestrone; oestradiol; oestradiol dipropionate and benzoate; stilboestrol	Subcutaneously by injection or implantation of pellets (dosage varied considerably); 2 days to 9 months	202/555	125–170 (using oestradiol benzoate)
Eisen (1942)	Inbred Sherman	Oestradiol dipropionate, 2 × 5 mg. in paraffin wax	Subcutaneously as pellets (a few only) or subcutaneously, intra-muscularly or intraperitoneally in liquid paraffin wax; 3–5 weeks	2/134	376
Chamorro (1943)	Unstated	Oestrone benzoate in oil, 50–100 μg.	Subcutaneously by injection; 3 weeks	6/10	609
Dunning *et al.* (1947)	A × C line 9935; Fischer line 344; Copenhagen line 2331	Stilboestrol; (*a*) 15–25 mg. pellet repeated, unsuccessful; (*b*) 4–15 mg. in Cholesterol (1 to 4) successful	Subcutaneously as pellets; 3–4 months	39/117 6/95 0/108	320
Nelson (1944)	Long-Evans hooded	Oestrone, 3 to 6 × 10 mg. pellets; stilboestrol by injection, 50μ daily, 1–4 × 10 mg. pellets	Subcutaneously by injection or implantation of pellets; 60–80 days	68/103	130 (using oestrone)
MacKenzie	WAG (albino) Newcastle (hooded); Cancer Hospital (albino)	Oestradiol, oestradiol dipropionate; stilboestrol; 2 × 6 mg. pellets	Intramuscular implantation of pellets; 28 days	10/46 8/33 3/61	230 (using oestradiol)

tumours. The difference between them lies in the method of absorption, which in the one case is discontinuous while in the other there is a continuous gradual absorption of the active substance, with the result that a considerably smaller total dose is required to produce the desired effect. Geschickter and Byrnes (1942) found that only one third to one half of the amount given by daily injection was required to produce mammary cancer if given in the form of pellet implants; in addition, the great saving in labour need not be stressed. With regard to the vehicle in which the active substance is given, when the injection technique is used it is dissolved in some oil, such as corn oil or olive oil. Pellets are made of the pure substance, but, as already noted, Dunning Curtis and Segaloff (1947) have demonstrated the value of mixing it with cholesterol when stilboestrol is being used. Eisen (1942), however, used oestradiol dipropionate dissolved in liquified paraffin which, when cooled in the body, formed a solid wax pellet from which the active substance was gradually absorbed. However, these pellets appeared to evoke a marked acellular connective tissue reaction around them and it is possible that the rate of absorbtion was much reduced, thus accounting for the low incidence of tumours which he obtained.

Duration of stimulation.—From Table V it will be seen that the time from the onset of oestrogenic stimulation to the appearance of palpable nodules of mammary cancer varies considerably. This may well be a product of three variables: (*a*) strain of rat, (*b*) type of oestrogen, and (*c*) dosage; and it is difficult to know what the relative importance of each factor may be. From the figures, however, it appears that palpable nodules are unlikely to appear in less than 120 days, though tumours may be discovered microscopically before that. Therefore any experiment must be continued either (*a*) until the appearance of a tumour or (*b*) until the death of the animal. In the present experiments the earliest tumour was noted about the 230th day after the beginning of oestrogenic stimulation, but others developed much later than this. Geschickter and Byrnes (1942) found that the time of appearance of a tumour depended largely on the dosage of oestrogen given, the larger the dose within a given limit (200μg. daily in the case of oestrone) the earlier the tumours appeared. Also they state that the older the animal is at the onset of stimulation the larger the dose must be to cause the the appearance of cancer. Amounts greater than the optimum did not decrease the time of appearance of the tumours, nor increase the number of animals with tumours.

SUMMARY.

1. Female rats of three different strains were implanted with oestradiol, oestradiol dipropionate, or stilboestrol, and kept under observation till they died.

2. Out of a total of 140 female animals, 21 developed malignant mammary tumours, some of them showing multiple tumours.

3. Four animals developed malignant tumours of the ovary or uterus.

4. The experimental conditions necessary to the production of malignant tumours of the breast in rats are discussed.

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PULMONARY TUMOURS IN URETHANE-TREATED MICE: FAILURE TO DEMONSTRATE AN UNDERLYING INFECTIVE AETIOLOGY.

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IT is well known that in many breeds of mice pulmonary adenomata occur spontaneously (Law, 1954). In some breeds the incidence may approach 100 per cent in older mice although the number of tumours borne by individual mice is usually low. Several of the carcinogenic hydrocarbons, appropriately administered increase the incidence of pulmonary adenomata in such susceptible strains of mice (Andervont and Shimkin, 1940). Gamma rays also induce the formation of these tumours (Lorenz *et al.*, 1946), and Rogers (1955) reported that adenomata develop in grafts of foetal lung previously irradiated *in vitro* with ultraviolet light. There are breeds, however, which have no natural incidence of pulmonary tumours, and in these it may be difficult or impossible to induce lung tumour-formation by chemical agents (Gross *et al.*, 1953).

The genetics of pulmonary tumours was first studied by Lynch (1926). Law (1954) reviews the now considerable literature on this subject. He concludes that susceptibility to lung tumours (both spontaneous and induced) is influenced by genes located on at least four chromosomes; and a relationship has been demonstrated (Heston *et al.*, 1952) between genes which lower the incidence of lung tumours and those which decrease body weight.

Nettleship and Henshaw (1943) were the first to describe the high incidence of pulmonary tumours in mice after treatment with urethane (ethyl carbamate). This finding, now amply confirmed, has been studied by many other workers. The induction of lung tumours by urethane does not depend on any particular route of administration. Intraperitoneal injection (Nettleship and Henshaw, 1943), oral administration (Selbie and Thackray, 1948), and application to the skin (Cowen, 1950) have all been shown to be effective. Moreover, induction by the transplacental route has been demonstrated: urethane administered to the pregnant female of a susceptible strain leads to a high incidence of lung tumours in the progeny (Larsen, 1947*a*).

Larsen (1947*b*, 1948) tested a series of carbamates related to urethane, and a number of alkylated urethanes. Ethyl N-isopropyl carbamate was found to possess moderate lung tumour-inducing activity, but none of the other compounds examined showed more than slight activity. Larsen (1950) also tested some of the possible breakdown-products of urethane, namely ethyl alcohol, carbon dioxide (in the form of sodium bicarbonate), ammonia (as ammonium carbonate and ammonium chloride), and cyanic acid (as potassium cyanate). He concluded that the effect observed following the administration of urethane was most probably due

to urethane itself and not to one of its breakdown products, and that within the series of related compounds tested the activity was almost specific for urethane.

Urethane possesses both antileukaemic and narcotic properties, and several substances which possess one of these properties have been tested for lung tumour-inducing action. Larsen, Rhoads and Weed (1946) examined a number of narcotic agents, but none showed activity. Shimkin (1954) tested a series of antileukaemic agents. He reported that apart from urethane both nitrogen mustard (HN2) and triethylene melamine (T.E.M.) were capable of increasing the incidence of lung tumours in mice. Aminopterin, Myleran, stilbamidine, Fowler's solution, and cortisone all failed to do so.

Despite the simplicity of the urethane molecule, the chemical mechanism(s) underlying its various pharmacological actions is almost a complete mystery. Todd (quoted by Haddow and Sexton, 1946) suggested that urethane may interfere with nucleotide synthesis. In support of this Cowen (1949) demonstrated that the induction of lung tumours by urethane was inhibited by the simultaneous administration of pentose nucleotides. Boyland (1952) thought that urethane might cause thymine-deficiency by prevention of the methylation of uracil to thymine. Later (Boyland and Koller, 1954), it was found that simultaneous administration of thymine reduced the incidence of abnormal mitosis due to urethane in the Walker rat carcinoma 256. Haddow and Sexton (1946) suggested that urethane may interfere with purine synthesis. There is evidence (Roe, 1955) that simultaneous administration of the purine precursors, glycine and formate, inhibits the initiation of skin tumour-formation by urethane (Salaman and Roe, 1953).

The observations of Noble and Millar (1948) cannot be fitted into the general background of work, briefly reviewed above, on the genetics and chemical induction of pulmonary tumours and, in particular, on the chemical basis of the action of urethane. These workers, in a preliminary report, recorded the occurrence of adenomata in the lungs of a small number of " Swiss " albino mice 6 months after the subcutaneous injection of a suspension of adenoma-containing lung tissue, taken from donor mice which had been treated with urethane not less than 5 months before. In another experiment mice were injected with lung tissue from donor mice which had been given injections of urethane 3, 6, or 24 hours previously. Single or multiple adenomata were seen in the lungs of the recipient mice at *post mortem* 6 months later. Control mice injected with lung tissue from untreated donors developed no tumours. It seems unlikely that sufficient urethane could have persisted in the donor lung to account for the subsequent development of lung adenomata in the hosts, in view of the findings of Bryan, Skipper and White (1949), Skipper *et al.* (1951), and Boyland and Rhoden (1949) who showed that at least 90 per cent of urethane injected into normal mice is metabolised in the first 24 hours after injection.

The experiment described below was designed as a repetition of the work of Noble and Millar (1948). A repetition was clearly desirable because of the difficulty of reconciling their results with the most generally held view of the aetiology of lung adenomata, namely that their occurrence depends on two factors only, a genetic susceptibility, and the action of extraneous chemical or physical agents. It has been suggested however (Selbie and Thackray, 1948) that an infective agent may also play a part in the production of lung adenomata in mice. A confirmation of Noble and Millar's results would strengthen this suggestion.

EXPERIMENTAL.

Male mice of the stock albino strain " S " (Salaman and Gwynn, 1951), aged approximately 8–10 weeks were treated as follows :

(*a*) Fifteen mice were injected subcutaneously, and a further 8 mice intraperitoneally, with a suspension of adenoma-bearing lung tissue from mice previously treated with urethane [3 to 18 applications of 0·3 ml. of a 20 per cent w/v solution of urethane in acetone (0·18 to 1·08 g.) to the skin]. Each recipient mouse was given the equivalent of one lung from a donor mouse, consisting of up to 50 per cent of adenomatous tissue. Before injection the lung was homogenized and suspended in Ringer solution. None of the donor mice had received treatment with urethane for at least 10 weeks before sacrifice.

Thirteen mice injected subcutaneously and 5 intraperitoneally survived until 6 months after injection. Of these 16 had no lung adenomata, 2 others bore one adenoma each. This incidence is well within the limits for untreated mice of the same age and strain.

(*b*) To control against a possible positive result in the above experiment being due to residual urethane in the injected lung tissue, three groups, each of 4 mice, were injected with the equivalent of one lung from donor mice which had received two injections of 20 mg. urethane in 0·4 ml. Ringer solution intraperitoneally 24 and 4 hours, 72 and 48 hours, and 8 and 7 days, respectively, before sacrifice. The lung homogenate was heated in a water bath at 100° C. for 15 minutes, before injection, in order to destroy any heat-sensitive agent while preserving unmetabolised urethane. (In a preliminary experiment it was shown that similar heat treatment of an aqueous solution of urethane (100° C. for 15 minutes) caused no loss of anaesthetic potency.)

A further group of 4 mice were injected intraperitoneally with heat-treated homogenized liver (equivalent of half a liver per recipient mouse) from donor mice injected twice with 20 mg. urethane (24 and 4 hours previously).

Six months later the 14 survivors of this experiment were killed, and *post mortem* examination revealed no adenomata in 12 of the mice, and one adenoma in each of the remaining 2. This incidence was well within the limits for untreated mice.

CONCLUSION.

These experiments, essentially similar to those of Noble and Millar (1948), but conducted in a different strain of mice, failed to confirm their results. The suggestion that an infective agent is concerned in the production of the lung adenomata which develop after urethane treatment is not supported by the present findings.

SUMMARY.

1. The experiments of Noble and Millar (1948), in which lung adenomata developed in mice injected with suspensions of adenomatous lung obtained from urethane-treated mice, were repeated in a different strain. Their results were not confirmed.

2. Noble and Millar's findings had suggested that an infective agent was concerned in the development of the lung adenomata which follow urethane treatment. This and other reports on the problem are discussed. It is concluded that no unequivocal evidence for an infective aetiology exists at present.

ADDENDUM.

During the preparation of this paper Professor R. L. Noble kindly communicated to us the results of experiments carried out subsequent to the publication of the preliminary report referred to in the text (Noble and Millar, 1948).

In the course of a repetition of their original experiments with much larger numbers of animals it was found that the " Swiss " albino strain used had a high incidence of spontaneous lung adenomata. None of the experimental procedures originally used increased this incidence significantly.

We thank Dr. M. H. Salaman for his advice. The expenses of this research were partly defrayed out of a Block Grant from the British Empire Cancer Campaign.

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3:4-BENZPYRENE AND OTHER POLYCYCLIC HYDROCARBONS IN CIGARETTE SMOKE.

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In a previous investigation (Commins, Cooper and Lindsey, 1954) the presence of anthracene and pyrene in tobacco smoke was demonstrated. Although 3 : 4-benzpyrene was not detected then, chromatographic fractions of neutral material exhibiting strong fluorescence were obtained in sequences where, from experience with control experiments with mixtures of pure hydrocarbons, this compound would be expected.

Experiments using larger amounts of material have now revealed small quantities of 3 : 4-benzpyrene in some such fractions (Cooper, Lindsey and Waller, 1954). In addition acenaphthylene, pyrene and anthracene were also detected and determined in cigarette smoke. A full description of this work follows.

Preparation of smoke.

The apparatus previously described proved to be inadequate to produce the larger quantities of condensed smoke needed in this investigation and so a new smoking device was designed to smoke a number of cigarettes at once and to conform to the conditions simulating normal human smoking. The essential parts of this apparatus are shown in Fig. 1.

A is a multiple holder to take 5 cigarettes, connected by means of a standard joint to two absorption flasks, B, of 100 ml. capacity. These in turn were joined to an absorption column, C, 2·5 cm. in diameter and 80 cm. long, filled with glass beads 0·5 cm. in diameter. Throughout the apparatus interchangeable standard joints were employed to facilitate replacement of units. C was connected to the intermittent suction device. An adaptor, D, could be employed to use two multiple holders and thus smoke 10 cigarettes at a time.

This design of apparatus can readily be modified for other types of experiment by connecting pipe-smoking units for cut tobaccos, filter units, electrostatic precipitating units, liquid air or solid CO_2 traps, etc.

In the experiments for this investigation the absorption vessels were charged with a layer of cyclohexane and advantage was thus taken of the fine state of division of the aerosol to effect the separation of some of the hydrocarbon soluble constituents. After considerable time a lower layer of hydrophilic condensate separated beneath the cyclohexane. The conditions of smoking were as previously described (Commins, Cooper and Lindsey, 1954).

Preparation of a solution of neutral constituents.

At the end of each experiment, in which 500 cigarettes were smoked, cyclohexane was drained off and the rest of the condensate washed out with acetone. To the clear acetone solution an equal volume of cyclohexane was added and the whole distilled in a waterbath until the temperature of the vapour reached about 70° C. The cyclohexane was then poured off from the viscous layer that had separated and the residue was again extracted by boiling with cyclohexane. The cyclohexane solutions were washed in turn with 2N sulphuric acid (3 times) water, 2N sodium hydroxide (3 times) and water. The neutral cyclohexane solutions were separately reduced to small bulk (a few ml.) by distillation and chromatographed on alumina columns by the technique described below.

Preliminary Procedure.

The spectrophotometric examination of cyclohexane extracts of tobacco smoke condensate for hydrocarbons has always presented great difficulty because of the

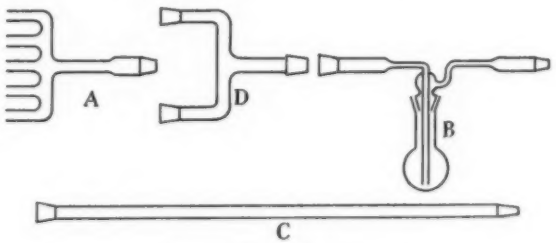

FIG. 1.—The smoking machine.

very considerable background absorption of ultra-violet radiation, and the method of repetitive chromatography is only possible if the fractions containing individual compounds can be identified and segregated for further chromatographic separation. Preliminary recognition of fractions is thus of prime importance.

The background absorption curve in the absence of a constituent giving a peak is usually less steep as the wavelength increases. In the presence of a substance too small in amount to give a peak, a change in direction of the curve may readily be detected by measuring the slope before and after the precise wave-length of the peak expected. This technique, applied to the 385 mμ peak of 3 : 4-benzpyrene, and aided by the characteristic fluorescence of the hydrocarbon, has been successful in segregating fractions for further treatment. The detailed technique is as follows.

The dark brown cyclohexane extract was passed through a large chromatographic column of alumina (10 cm. $\times$ 2·8 cm.), the bottom half of the column being composed of alumina of fairly low activity and the top half containing alumina of high activity. This was in effect a means of shortening a column of low activity. Most of the strongly adsorbed material was thus held as a dark brown zone in the top part of the column, while the hydrocarbons and other less strongly adsorbed substances were spread out in the lower half as zones of various light coloured constituents.

The first zone to be eluted with cyclohexane was a pale bluish-green which showed a high optical density in the region 270 to 300 mμ. The subsequent colourless and light yellow zones showed a violet fluorescence similar to that of the polynuclear hydrocarbons. The deeper yellow one above these showed a blue fluorescence at the lower edge only, fluorescence above this being quenched by strong absorption of light. After the first zone had passed through, the column eluates were examined for specific peaks, beginning with that for acenaphthylene at 340 mμ. At the same time examination was made for the anthracene peak at 376 mμ., where the background absorption was much lower than that at 340 mμ. All the ten or so fractions showing a peak or even an inflexion at 340 mμ. were collected together and transferred to a stoppered glass vessel for subsequent examination. Similarly all eluates showing peaks and inflexions at 335 mμ. and 376 mμ. for pyrene and anthracene respectively were set aside as separate fractions. The column was allowed to develop until the optical density of the eluates between 350 and 400 mμ. began to diminish considerably, showing that the cyclohexane was no longer eluting material from the column. All such eluates were tested for 3 : 4-benzpyrene, by the method later described, until it was certain that this compound, if present, was still on the column. At this stage examination of the column under filtered ultra-violet light revealed a violet fluorescence just above the base of the column, with a bright blue fluorescence above this passing into the yellow zone. The zone showing violet fluorescence was suspected of containing the benzpyrene. This was then washed out of the column by the addition of a small proportion of benzene to the cyclohexane used for elution. When the whole of this had passed through and a little of the material in the higher zone had been allowed to follow it, these eluates were combined for further treatment. Pure benzene was next added to the column and a great deal of the yellow coloured material washed out of the column and collected as a separate fraction. Finally the column was washed through with chloroform, which thus yielded a dark orange-brown fraction. This also was kept as a separate fraction. Although some material was still retained on the column, it was thrown away when the alumina was removed.

Analysis of the Chromatographically Separated Fractions.

(a) *The fraction suspected of containing* 3 : 4*-benzpyrene.*

The solution in benzene and cyclohexane was distilled to dryness and the residue taken up in boiling cyclohexane. This procedure was repeated twice and finally a pale yellow solution with violet fluorescence was obtained in about 5 ml. of cyclohexane free from benzene. This was passed through a small column of alumina (2·5 cm. × 1·4 cm.) of very low activity. As the development of the column proceeded most of the yellow substances were retained at the top, while only very pale yellow zones slowly spread down the alumina. The leading zone showed a violet fluorescence in ultra-violet light and contained 3 : 4-benzpyrene in amounts so small that it could only be recognised by an inflexion at the wave-length 385 mμ. This inflexion was most readily found by comparing the differences in optical densities at the wave-lengths 377·5, 385 and 392·5 mμ. By passing the combined filtrates through further short columns of alumina some improvement of the inflexion was obtained. At this stage silica gel was employed as a column material to remove much of the remaining background, which was held as a yellow

zone near the top while the violet fluorescent zone passed rapidly through in a few eluates. The 3 : 4-benzpyrene was revealed in these eluates by peaks at 385 mμ. and 403 mμ. and a strong inflexion at 365 mμ. and was then determined in a known volume of the cyclohexane solution by peak height measurement.

This solution was also examined by the method of fluorescence spectrography and the presence of 3 : 4-benzpyrene confirmed. Of the various polycyclic hydrocarbons separating closely together in alumina chromatography, 3 : 4-benzpyrene is clearly recognisable by its fluorescence spectrum (Waller, 1952). 1 : 12-Benzperylene with which it is associated does not interfere.

(b) *Other fractions containing hydrocarbons.*

The fraction believed to contain acenaphthylene was passed through a fresh column of alumina and the separation improved using the peak at 340 mμ. as a guide. As this became clearer against the background, so two other peaks appeared corresponding to the peaks of acenaphthylene at 324 mμ. and 334 mμ. In a similar manner anthracene was separated as revealed by its peaks at 340 mμ., 357 mμ. and 376 mμ., and pyrene by peaks at 273 mμ., 319 mμ. and 335 mμ. These were also determined by peak height measurement. Other compounds also detected, but not determined, were fluoranthene, anthanthrene, an alkyl pyrene and what appeared to be an alkyl anthracene, the latter showing peaks at 359 mμ. and 378 mμ. Peaks of other compounds also appeared at 294 mμ. and 300 mμ., but these were not identified.

Examination of Denicotea Filters.

In addition to using material from the smoking machine, the well-known proprietary " Denicotea " filters were used to provide a smoke condensate for analysis. These filters contain a colourless transparent filter material in a cylindrical plastic casing which fits a special cigarette holder. The filter is thus interposed between the cigarette and the smoker's mouth. Each filter was used for about 10 cigarettes.

An advantage of using a filter as a source of material is that the process, being actual human smoking, is not " artificial ". A disadvantage is that quantitative results refer only to the unknown amount of the total smoke condensed in the filter, and that if the filter exerts a selective action the results refer only to the substances retained. A further disadvantage is that the filters, collected by the co-operation of various smokers, may be exposed to chance contamination before being collected for analysis.

The smoke adsorbed on the filter medium was removed by extraction with cyclohexane in a Soxhlet apparatus. As many as 170 could be conveniently extracted at a time. Preliminary experiments have shown that, although cyclohexane extracts only a small fraction of the total material present in the filters, almost the whole of the polycyclic hydrocarbons are removed by this solvent. Acetone, which removes much more of the total material than cyclohexane, extracts only a small amount more of the polycyclic hydrocarbons. Although the filters are still deeply coloured after treatment with acetone, no more than minute traces of polycyclic hydrocarbons could be detected in the finely ground filters after this treatment. It thus appears that the hydrocarbons are not held firmly by the filters and do not penetrate to any considerable extent into the interior.

The cyclohexane extracts from filters are less strongly absorbing for ultra-violet light than the extracts obtained from the smoking machine and thus the hydrocarbons present are more easy to segregate and determine by the methods outlined above. Acenaphthylene, anthracene, pyrene, 3 : 4-benzpyrene and 1 : 12-benzperylene were found, with traces of fluoranthene, phenanthrene, anthanthrene, an alkyl pyrene and an alkyl naphthalene.

Analytical Results.

In the analysis of cigarette smoke, a popular brand of cigarette made and sold in this country was smoked in the machine. The figures quoted in Table I refer to 500 cigarettes consumed to a butt of about 1·5 cm. and correspond to about 440 g. tobacco burnt.

TABLE I.—*Analysis of Smoke from 500 Cigarettes.*

Acenaphthylene	20·5 micrograms
Anthracene	48·0 ,,
Pyrene	55·0 ,,
3 : 4-Benzpyrene	4·0 ,,
1 : 12-Benzperylene	0·5 ,,
Fluoranthene, Phenanthrene, Anthanthrene	Traces, amounts yet to be confirmed

In the analysis of extracts from "Denicotea" filters a great variation in the amounts of anthracene was found. This is clearly shown in Table II.

TABLE II.—*Analysis of Extract from "Denicotea."*

Number of filters.	Micrograms per 500 filters. Anthracene.	Pyrene.	3 : 4-Benzpyrene.
80	36·0	25·0	1·3
480	17·5	22·0	1·5
430	6·0	19·0	2·0
Mean	20·0	22·0	1·6

The amount of tobacco smoke drawn over these filters is variable. The filters were changed approximately every 10 cigarettes, so that 500 filters correspond roughly to 5000 cigarettes. It is therefore clear that the filters retain only a fraction of the polycyclic hydrocarbons present in the smoke and that the proportions are roughly those found in the whole smoke. The loss of anthracene indicated in the last experiment may be due to its oxidation in the time lapsing between the using of the filters and their analysis.

Blank Determinations.

The solvents were purified as previously described and a blank determination was carried out with each smoke analysis using all the reagents, including the water employed for washing. The glass apparatus employed was cleaned by immersion overnight in chromic-sulphuric acid mixture, washing and drying.

Forty litres of air were aspirated through the smoking apparatus, using the intermittent suction employed in its use. No compounds other than pyrene (0·02 μg.) could be detected.

Blank determinations were also carried out on 500 unused filters, 76 g. alumina and 35 g. silica gel as used in the experimental procedure. The results in micrograms were as shown in Table III.

TABLE III.—*Analysis of Unused Filters, Alumina and Silica.*

	Anthracene.	Pyrene.	3 : 4-Benzpyrene.
Unused filters . . .	0	0·8	0·3
Alumina	0	0·04	0
Silica	0	0·07	0·03

From these results it will be seen that the analyses of smoke condensates are not significantly affected by the blank determinations.

DISCUSSION.

A previous reference to the presence of polycyclic hydrocarbons in tobacco smoke was made by Ikeda (1947), who reported the presence of azulene in the product obtained by distilling condensed smoke. We have not yet confirmed its presence, but believe it to be in the green fraction which emerges first from our chromatographic columns.

Although there is now an extensive literature upon the effects of tobacco smoke upon mammals (see Wynder, Graham and Croninger (1953) for a bibliography of 42 references), no specific organic carcinogen has previously been shown to be present in such smoke. 3 : 4-Benzpyrene and 1 : 12-benzperylene are both carcinogenic, but the latter only feebly so (Kennaway, 1954, private communication).

SUMMARY.

Tobacco smoke obtained by smoking cigarettes in a machine to simulate normal human smoking has been shown to contain acenaphthylene, anthracene, pyrene, 3 : 4-benzpyrene, 1 : 12-benzperylene and traces of fluoranthene, phenanthrene, anthanthrene, and possibly 3 methyl pyrene, 2 methyl anthracene and 2 methyl naphthalene. Most of these hydrocarbons were also shown to be present in the smoke trapped by Denicotea filters.

The amount of benzpyrene in the smoke from 500 cigarettes was 4·0 μg.

The authors wish to acknowledge the helpful criticism given by Professor Sir E. L. Kennaway, F.R.S., and Professor J. W. Cook, F.R.S. They also wish to thank Mr. R. E. Waller for his confirmation of the presence of 3 : 4-benzpyrene by fluorescence spectrography, and the Medical Research Council for supporting the investigation.

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RETARDATION OF AZO-CARCINOGENESIS BY NON-CARCINOGENIC AZO-COMPOUNDS.

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THE capacity of certain azo-compounds to induce liver tumours is partly determined by their chemical constitution, but no comprehensive correlation between structure and carcinogenic power has yet emerged. This variation in potency was clearly demonstrated by feeding six isomeric aminoazotoluenes, incorporated in the same basal semisynthetic diet, to rats and mice (Crabtree, 1949). All but one of these isomers proved to be carcinogenic for mice, but only two of them induced hepatomas in rats. In an attempt to assess the significance of these results, particularly those found for rats, the possible rôle of p-aminobenzoic acid (p-AB) was emphasised. When the parent molecule was a potential precursor of p-AB the rate of growth of the animals was notably enhanced and tumour development did not occur. Conversely, the liberation of o-aminobenzoic acid was accompanied by retarded growth and the induction of liver tumours. Since the same diamine was formed in both these cases it was suggested that the potentiality of liberating p-AB might be one factor determining the genesis of this type of tumour.

These observations have been extended in two ways. First, by testing the carcinogenicity of azo-compounds containing the potential p-AB within the parent molecule and, secondly, by studying the action on a known azo-carcinogen of another azo-compound, itself non-carcinogenic but acting as a potential precursor of p-AB. For this purpose five compounds have been prepared and shown to possess these desired properties. They have been incorporated together with o-aminoazotoluene or p-dimethyl-aminoazobenzene in the basic diet. This report describes their common, but variable, inhibitory action on liver-tumour induction.

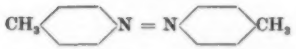
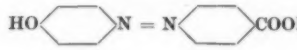
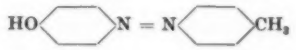
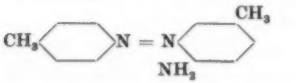
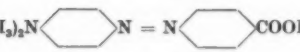
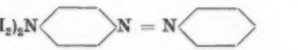
Since it has been shown that the addition of p-AB itself to the nutritionally incomplete diet used does not affect the rate of azo-carcinogenesis, the mechanism whereby the azo-compounds which are precursors of p-AB exert their effect is at present obscure.

MATERIALS AND METHODS.

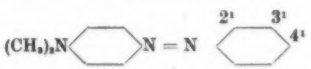
Animals.—Rats of the Glaxo strain were used in all experiments, males having an initial average weight of 150–165 g., and females 130–140 g. The sexes were housed separately and fed on the basic semisynthetic diet with appropriate addition of azo-compounds as described previously (Crabtree, 1949).

Azo-compounds used.—Table I gives details of the azo-compounds used in the experiments where the carcinogen was fed in conjunction with one of the precursors of p-AB, *i.e.* P1, P2, P3, P4, or P5,

TABLE I.

Symbol.	Formula.	Name.
P1		4 : 4¹-azotoluene.
P2		4-hydroxy-azobenzene-4¹-carboxylic acid.
P3		4-hydroxy-4¹-methyl-azobenzene.
P4		2¹-amino-4 : 5¹-azotoluene.
P5		4-dimethylamino-azobenzene-4¹-carboxylic acid.
BY		4-dimethylamino-azobenzene (butter yellow)

In order to study the effect of introducing within a carcinogenic molecule a substituent ensuring the liberation of p-AB together with an " active " diamine, three isomeric azo-compounds consisting of BY modified by the presence of a carboxy group in the 2^1, 3^1, or 4^1 position were tested for carcinogenic activity.



Preparation of azo-compounds.

P1 : Prepared by the method of Perkin (1880). p-toluene in EtOH-solution was reduced by 5 per cent sodium amalgam. The purest product was obtained by keeping the temperature not higher than 30°. The broth of crystals, after filtration, gave pale orange needles from glacial acetic acid or ligroin. m.p. 144°.

P2 : Diazotised p-AB was coupled with phenol in alkaline solution at 0–5° and the product precipitated by acetic acid. Crystallisation from EtOH yielded dark red needles. m.p. 278° (decomp.).

P3 : Diazotised p-toluidine was coupled with phenol in alkaline solution at 0–5°. The filtered yellow precipitate gave a crystalline product from EtOH. m.p. 140° (decomp.).

P4 : Prepared as previously described (Crabtree, 1949).

P5 : Diazotised p-AB was coupled with dimethylaniline hydrochloride in the presence of excess sodium acetate. The vermilion precipitate was crystallised from glacial acetic acid, giving minute rods. m.p. 232° (decomp.).

Isomers of P5.

(*a*) *2^1-carboxylic acid.*—Diazotised o-aminobenzoic acid was coupled with dimethylaniline by the method used for P5. The rate of coupling was much slower. From EtOH bright red crystalline fibres were obtained. m.p. 180° (decomp.).

(*b*) *3^1-carboxylic acid.*—From m-aminobenzoic acid and dimethylaniline as (*a*). Crystalline masses from EtOH. m.p. 206° (decomp.).

All these compounds were fissioned by reduction with Sn + HCl, and the products were identified by isolation or special tests.

METABOLISM OF P1, P2, P3, P4 AND P5.

The end products of metabolism, found in the urine of rats consuming these substances, were studied by analytical methods and by the preparation of characteristic derivatives.

Six rats, in metabolism cages, consumed powdered rat cubes containing 1·2 per cent of the compound under test and estimations were carried out daily for ten days. Normal values had been measured previously.

The scheme of analysis used by Bray and Thorpe (1948) for investigation of the metabolism of acetotoluidines was adopted, in which changes in the diazotisable constituents, reducing substances, ethereal sulphate and ether-soluble acids were assessed.

Diazotisable constituents.

In theory, all substances containing an aminogroup are estimated by this procedure. The amino-compounds which could be liberated by the breakdown of P1, P2, P3, P4 and P5 include p-aminophenol (as glucuronide or ethereal sulphate) p-toluidine, p-AB, 3:4-toluylene diamine and N,N-dimethyl-p-phenylene-diamine.

Under the experimental conditions used (Bratton and Marshall, 1939) p-AB alone was almost entirely responsible for colour development. The two diamines contributed little or no colour value to the solutions, p-toluidine was (except for very small amounts in the cases of P1 and P4) not detectable in the urines, and aminophenol coupled very slowly, interfering only slightly with the maximum colour-depth attained by p-AB in 10–15 minutes.

These estimations of p-AB were carried out on hydrolysed and unhydrolysed urines.

Reducing substances.

The method of Shaffer-Hartman was used (Peters and van Slyke, 1932). It measures the amount of p-AB conjugated as ester-glucuronide.

Ethereal sulphate.

Method of Folin (1905–6). Since only phenolic substances are excreted as esters of sulphuric acid, the occurrence of aminophenol in the urine would mainly account for the increased values found.

Ether-soluble acids.

These estimations were done on unhydrolysed urines using the method of Bray, Neale and Thorpe (1946). The extractions were made on acidified urines and measured the amount of p-acetyl-aminobenzoic acid formed.

The complete results of these analyses are collected in Table II. They show that all these azo-compounds undergo reductive fission and, in particular, prove to be precursors of p-AB. A rough calculation, based on the amount of food consumed, showed that over 90 per cent of P2 and P3 were metabolised in this way, though this high degree of breakdown was not attained in other cases.

TABLE II.—*Metabolism of Azo-compounds. Quantitative Increases in Constituents of Rat Urine after Feeding P1, P2, P3, P4, or P5.*

Azo-compound.	Ethereal sulphates. (%)	Reducing substances (as glucuronic acid). (%)	Diazotisable substances (as p-aminobenzoic acid). Free.	Diazotisable substances (as p-aminobenzoic acid). Total.	Ether-soluble acids of unhydrolysed urine.
P1	22	22	4-fold	25-fold	10-fold
P2	240	56	70- „	110- „	83- „
P3	160	82	57- „	90- „	43- „
P4	30	105	21- „	95- „	50- „
P5	50	75	32- „	80- „	40- „

Isolation of Split-Products.

P1: 500 ml. of urine + 50 ml. 4N HCl. were boiled for 30 minutes, made alkaline with NaOH and steam-distilled.

Distillate.—This was acidified with HCl., diazotised and coupled with β-naphthol. The azo-compound was reduced in 50 per cent EtOH by Sn + HCl., and the solution alkalised and steam-distilled. *p-toluidine* was identified in the distillate by colour tests and a very small amount of its acetyl derivative was prepared.

Residue.—After neutralisation with HCl, and evaporation to about 100 ml., it was brought to pH 4 by the addition of 100 ml. of Na_2HPO_4 + $NaHSO_4$ buffer (Bray *et al.*, 1948) and extracted with ether for 12 hours. The extracted substance, twice crystallised from H_2O, gave long colourless needles of p-AB, m.p. 186°, from which the mono-acetyl derivative, m.p. 258°, was prepared.

P2: 450 ml. of urine were boiled with HCl, alkalised with solid $NaHCO_3$, and, after adding a little $Na_2S_2O_4$ were extracted with ether for 10 hours.

Ether extract.—The residue, after removal of ether, was twice crystallised from H_2O, giving *p-aminophenol*, m.p. 184° (decomp.), from which the acetyl derivative and quinone were prepared.

Aqueous solution.—Treated as described for P1, gave *p-AB*.

P3: *p-aminophenol* and *p-AB* were isolated and identified by the methods used for P2.

P4: A small amount of *p-toluidine* and much *p-AB* were isolated by the methods used for P1.

Separation of 3 : 4-toluylene diamine: 500 ml. of urine were boiled with HCl, brought to pH 8 by the addition of solid $NaHCO_3$, and extracted for 12 hours with ether. The ethereal solution was dried with anhydrous Na_2SO_4, the ether removed, and the residue dissolved in dilute HCl and dried *in vacuo*. The diamine hydrochloride gave a diacetyl derivative, crystallised from H_2O, of m.p. 210°.

P5: *p-AB* was isolated from urine by the method used for P1.

No p-diamine was separated in pure condition, but the method used to isolate 3:4-toluylene diamine yielded a brown solid which showed the reactions of a p-diamine.

ANIMAL EXPERIMENTS.

1. *Azo-compounds Containing the Potential Aminobenzoic Acid within the Parent Molecule.*

The compound chosen for test was 4-dimethylamino-azobenzene-4^1-carboxylic acid, which on metabolic fission yields p-AB and a diamine identical with that formed from BY. The corresponding 2^1- and 3^1-carboxylic acids were also

fed for comparative purposes. The action of the 2^1-compound (" Methyl Red ") had been studied by Kinosita (1936, 1937) with conflicting results. Ingested by rats in olive oil solution it produced liver tumours and bladder papillomas in 1936, but no tumours in a second experiment in 1937.

None of these isomeric azo-compounds possessed the high carcinogenic activity of the parent substance BY. When each was fed, at a concentration of 0·06 per cent, to groups of 16 rats, one gross liver tumour was produced by the 2^1-derivative, two by the 3^1-derivative and none by the 4^1-derivative, during 400–600 days. Livers showing microscopic pathological changes were commonly found in the 2^1- and 3^1-groups, but the livers of rats of the 4^1-groups, with few exceptions, were almost normal in appearance. The rats in this latter group survived longer and were manifestly in better condition than those of the other groups; their relatively high rate of growth is shown in Fig. 1 (P5). Owing to intercurrent deaths due to lung infections the changes of growth of the 2^1- and 3^1-groups were difficult to assess, but the animals were in poorer condition and failed to maintain their initial rate of growth.

These results harmonise with those reviewed earlier (Crabtree, 1949) where several workers found that a methyl group in the 4^1-position of 4-dimethylaminoazobenzene or 4-methylaminoazobenzene caused an enormous fall in their carcinogenic potency. These Me-substituted carcinogens, like the 4^1-compound used in the present experiments, are also capable of yielding p-AB by reductive fission and oxidation and this common biochemical feature may well be the cause of their similar biological behaviour.

2. *BY in Conjunction with Precursors of p-AB.*

In the previous section experiments have been described which show that a modification of the molecule of the carcinogen BY by the introduction of a 4^1-methyl or 4^1-carboxy group produces molecules with little or no carcinogenic power and this change in biological properties has been attributed hypothetically to a release of p-AB on metabolic breakdown.

The possibility of antagonising the potency of BY by the simultaneous feeding of other azo-compounds capable of liberating p-AB in the liver—the site of metabolic attack on azo-compounds—was then investigated. The azo-compounds chosen for this purpose were not only precursors of p-AB, but were themselves non-carcinogenic. The latter property, initially presumed on the basis of existing data, was later demonstrated experimentally. The five azo-compounds used—P1, P2, P3, P4, and P5—had, with the exception of P5, the simplest possible structures yielding innocuous breakdown products, thus avoiding, as far as possible, complicating factors which might lead to uncertainty in the interpretation of results. Two of them, P2 and P3, proved to be relatively more toxic than the others, possibly because their polar groups facilitated attachment to proteins and interference with normal enzyme function.

(a) *Effect of P1, P2, P3, P4, and P5 on the growth of rats.*

Compound P4, known to be non-carcinogenic, had been shown to enhance the growth-rate of rats when included in the standard semi-synthetic diet and this property was attributed to the liberation of p-AB by metabolic processes (Crabtree, 1949). Since all these compounds possess this potentiality, their effect on growth,

when included in the basal diet at a concentration of 0·04 per cent, was investigated. The results are shown in Fig. 1.

Rats grew more rapidly when consuming any one of these substances, but not all to the same extent. There was no clear correlation between the degree of growth-stimulation produced by these compounds and their rate of metabolic breakdown or their solubility in fat-solvents. No doubt many factors contribute towards their nett effects on growth, but the outstanding common property is that of acting as precursors of p-AB.

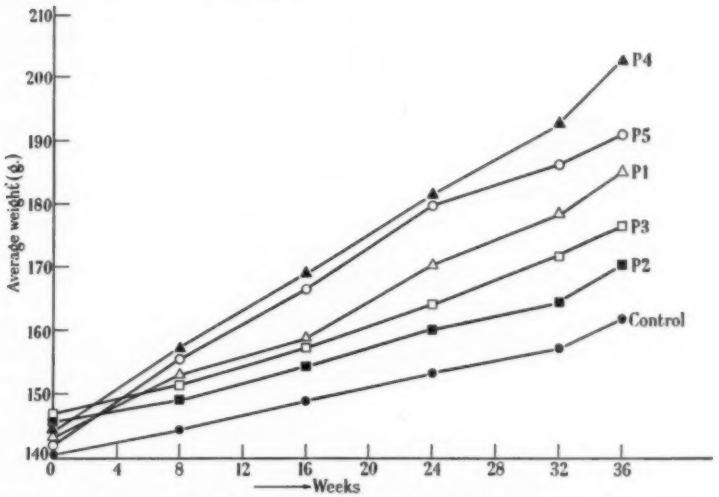


FIG. 1.—Growth curves of rats fed on the basal diet alone (control), or with the addition of 0·04 per cent of one of the five azo-compounds (P1, P2, P3, P4, P5) which yield p-amino-benzoic acid on metabolic breakdown.

(b) *Effect of P1, P2, P3, P4, and P5, on the growth of rats consuming BY.*

In studying the possible anti-carcinogenic power of these azo-compounds it became evident that their addition to a BY-containing diet influenced the rate of growth and the general condition of the rats.

The basal diet alone permitted a slow increase of weight, but when supplemented by 0·04 per cent of BY a gradual fall in weight occurred and continued until the emergence of liver tumours caused death at an average time of 186 days.

By contrast, diets containing BY together with 0·04 per cent of any one of the substances P1, P2, P3, P4, or P5 promoted the growth of the rats in variable degree, but in all cases the growth-depressing effect of BY was antagonised. Three groups of rats, P1 + BY, P2 + BY, and P4 + BY suffered early losses from lung infections, but the remaining animals continued to grow steadily, as is shown in Fig. 2.

(c) *Effects of P1, P2, P3, P4 and P5 on the induction of liver tumours by BY.*

Six groups of 16 rats were fed on the basic diet containing 0·04 per cent BY and in five groups a further supplement of 0·04 per cent of P1, P2, P3, P4 or P5

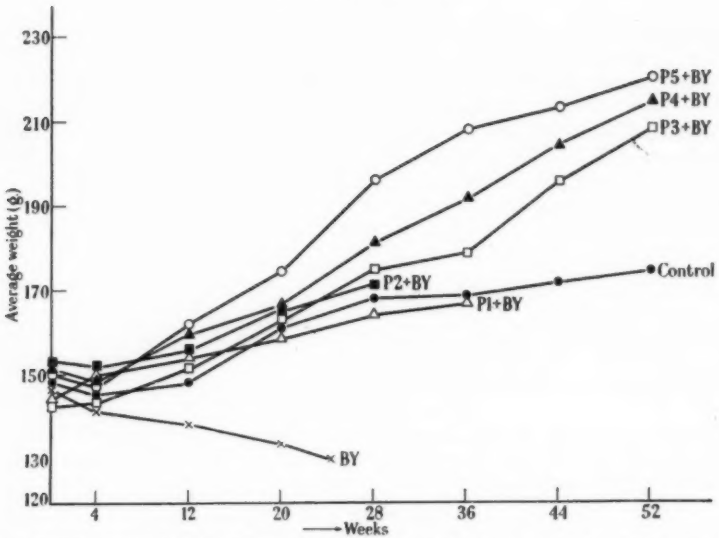


FIG. 2.—Growth curves of rats fed on the basal diet. (*a*) Alone (control). (*b*) Containing 0·04 per cent BY. (*c*) Containing 0·04 per cent BY and 0·04 per cent of one of the five azo-compounds (P1, P2, P3, P4, P5) which yield p-aminobenzoic acid on metabolic breakdown.

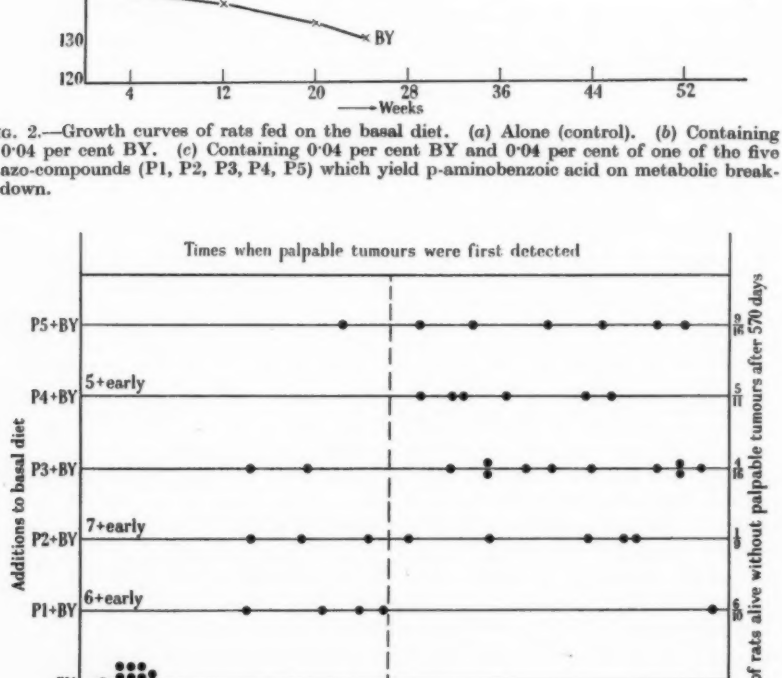


FIG. 3.—Showing the retarding effect of azo-compounds which yield p-aminobenzoic acid on metabolic breakdown (P1, P2, P3, P4, P5) on the rate of induction of liver tumours by butter yellow (BY) fed for 350 days.

was incorporated into the diet. The groups are referred to as BY, P1 + BY, P2 + BY, P3 + BY, P4 + BY and P5 + BY, three of which (Fig. 3) were reduced in numbers by infections at an early stage.

The emergence of liver tumours was easily detectable by palpation. From the size of a pea the growth of an out-growing nodule could be followed over 3–4 weeks, when a 20–30 g. tumour signalled the approaching death of the rat.

The time of occurrence of these small nodules is recorded in Fig. 3, each rat receiving a single mark. Early stages of malignant transformation were therefore not assessed, but since the process of nodule formation with subsequent enlargment was a common factor in all rats, the marks give an approximate measure of the rate of incidence of tumours.

After 200 days, all rats consuming BY alone carried tumours, some large and others just palpable, and were killed or died at an average time of 186 days. At this time no palpable tumour was found in any of the five remaining groups, and they were maintained until the 350th day on their respective diets, except that during this latter period they were twice given ordinary laboratory food for one to two weeks to maintain health and thus possibly prolong the inhibitory action already demonstrated. Some tumours emerged during this period, and after 350 days, the BY-feeding was discontinued, but the precursors of p-AB were still fed at the reduced rate of 0·02 per cent, until the 570th day, when all surviving animals were killed. Twenty-five rats, out of an original sixty-two, were still free from palpable tumours, though the livers of most of these animals were not normal. The detailed results are gathered together in Fig. 3. It is notable that the survival time of some of these rats was longer than that of animals consuming the basal diet alone, suggesting that the deficiencies of this diet are partially compensated by the azo-compounds which function as precursors for p-AB.

3. *Experiment with O-aminoazotoluene.*

The experimental procedures were similar to those described for BY except that 0·04 per cent o-aminoazotoluene (O : O) was the carcinogen used. During the first year the average weight of rats consuming O : O alone increased by 25 per cent, while an increase of 34–46 per cent occurred in rats consuming the carcinogen supplemented by 0·04 per cent of P1, P2, P3, or P4.

From this time onwards the general health of some of the animals tended to deteriorate slowly, and in some cases slight weight losses were observed. In order to promote longer survival all groups of rats were fed on ordinary laboratory diet for two spells of fourteen days during the second year of the experiments. Each rat was killed when a liver tumour was palpable or when signs of ill-health were evident, and all survivors were killed after 715 days of treatment.

A summary of results is shown in Table III. The long period necessary for the induction of liver-tumours by O : O represents a large fraction of the life-span of rats, and this increases the difficulty of demonstrating any inhibitory effects which may result from the feeding of additional non-carcinogenic azo-compounds. The average time of survival, though somewhat longer in the groups consuming supplements of P1, P2, P3, or P4, does not adequately reflect the degree of inhibition of the rate of tumour induction. For this reason the results are classified under the three headings shown in Table III. When no gross tumour was apparent and no minute tumour foci were visible, the liver was described as

"normal" regardless of the well-known cellular changes which precede the emergence of tumours.

TABLE III.—*Induction of Liver Tumours by O-Aminoazotoluene (O : O) Fed in Conjunction with P1, P2, P3, or P4.*

Azo-compounds.	Visible tumours.	Minute tumour foci.	"Normal" liver.	Days of treatment until death. Range (days).	Average (days).
O : O	12	2	2	522–670	600
O : O + P1	4	5	7	562–715	643
O : O + P2	2	4	10	531–715	626
O : O + P3	2	6	8	560–715	646
O : O + P4	5	5	6	529–715	637

Note on Method of Estimating the Times of Induction of Tumours.

The criteria used by different workers for comparing the carcinogenic activity of substances which induce liver tumours have varied. In many cases records seem to refer to the time of emergence of large tumours and in other cases the effects were assessed by killing all the animals at some arbitrary time. Laparotomies performed before tumours were palpable have sometimes provided additional information. Unless the differences between the experimental groups are wide, the interpretation of results may still be equivocal. Perhaps an experiment in which large numbers of animals were used and random samples killed at short intervals would be the ideal.

The criterion used in this work has been the time of emergence of small palpable tumour nodules. Though all early changes are thus ignored, this baseline seems justifiable on the grounds that it is a common event in almost all rats, and that the subsequent growth of the tumour is rapid and, in a short time, lethal. In any case, the general condition of the animals provides a fair indication of differential effects and the terminal stages of loss of weight and tumour enlargement occupy a relatively small part of the experimental period.

With these reservations in mind the conclusion seems justified that inhibitions in the rate of tumour induction by BY and O : O are a sequel of the additional feeding of precursors of p-AB.

SUMMARY.

1. Five azo-compounds,—P1, P2, P3, P4, and P5—which are potential precursors of p-aminobenzoic acid have been tested for anticarcinogenic action on 4-dimethyl-aminoazobenzene (BY). Their preparation and fate in the rat are described.

2. Rats consuming a basic diet containing 0·04 per cent of any one of these azo-compounds grew more quickly than rats fed on the basic diet alone. This augmentation of growth rate varied in degree with the compound used.

3. Rats on the same basic diet containing 0·04 per cent of BY progressively lost weight and liver tumours developed in all of them over a period of 200 days.

4. Rats consuming the BY-containing diet further supplemented with 0·04 per cent of any one of the compounds P1, P2, P3, P4, or P5 increased in weight,

and the rate of incidence of liver tumours was retarded in all cases to a variable degree.

5. In parallel experiments in which o-aminoazotoluene was the carcinogen used, a similar lowered incidence of liver tumours was demonstrated.

I am indebted to the late Dr. E. Vazquez-Lopez for generous collaboration in this and other work.

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THE ADSORPTION OF 3 : 4-BENZPYRENE AND ITS FLUORESCENT METABOLITES ON SERUM PROTEINS.

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STUDIES of the mechanism of elimination of 3 : 4-benzpyrene, 1 : 2 : 5 : 6-dibenzanthracene, and anthracene from the blood stream of injected animals (Peacock, 1936 ; Chalmers and Peacock, 1936) showed that 0 to 15 minutes after injection of benzpyrene colloid the hydrocarbon could be observed partly as yellow fluorescent particles showing Brownian movement and partly in violet fluorescent solution in the animals' body fat. Later, after 15 to 30 minutes, the number of fluorescent particles decreased and the intensity of fluorescence of the body fat increased. During the subsequent period, 30 minutes to 2 hours, no fluorescent particles were seen in the blood, the intensity of fluorescence of the body fat decreased and fluorescent bile appeared in the gall bladder reaching a maximum after 1 to 2 hours. After 2 to 6 hours the abnormal fluorescence of the body fat disappeared first and subsequently the blue fluorescence of the bile disappeared.

After intravenous injection benzpyrene is eliminated in metabolised form chiefly in the faeces although a significant amount is excreted in the urine (about 20 per cent) (Heidelberger and Weiss, 1951). A fraction of the order of 1 per cent of the injected hydrocarbon can be recovered unchanged from the faeces of rats and mice (Chalmers and Kirby, 1940 ; Berenblum and Schoental, 1942). Owing to their instability the recovery of metabolic products of benzpyrene from the faeces of injected animals presents some difficulty. A small quantity of a monohydroxy benzpyrene was however isolated from the faeces of rats (Chalmers and Crowfoot, 1941). This substance was identified as 8-hydroxybenzpyrene by Berenblum and Schoental (1943), Berenblum, Crowfoot, Holiday and Schoental (1943) and subsequently synthesised by Cook, Ludwiczak and Schoental (1950). In addition to the 8-hydroxy compound the faeces of injected rats also contain 10-hydroxybenzpyrene and the oxidation products of these two phenols, 3,4-benzpyrene 5,8-quinone and 3,4-benzpyrene 5,10-quinone, (Berenblum and Schoental 1946).

Four fluorescent metabolic products of benzpyrene have been separated from the tissues of treated animals by Doniach, Mottram and Weigert (1943), Weigert and Mottram (1946*a*, 1946*b*), Weigert, Calcutt and Powell (1947). In the first of these papers, which deals with the distribution of fluorescence at various sites, reference is made to the precipitation with ammonium sulphate of blue fluorescent material with the globulin fraction of an alkaline extract of mouse skin after painting with benzpyrene. Later Miller (1951) gave evidence of an *in vivo* binding of benzpyrene with skin protein which could not be obtained *in vitro*. This author made a preparation of precipitated skin protein and nucleoprotein

which was extracted with hot ethanol and then hydrolysed to liberate the bound hydrocarbon. Wiest and Heidelberger (1953*a*) in tracer studies with 1,2,5,6-dibenzanthracene-9,10-C^{14} confirmed this work as also did Woodhouse (1954) who, in addition, found that non-carcinogenic hydrocarbons were also bound to skin proteins in the same way. *In vivo* and also *in vitro* binding of 1,2,5,6-dibenzanthracene-9,10-C^{14} with the proteins of the mouse submaxillary gland which contrasts with the *in vivo* binding of this compound with the proteins of mouse skin was described by Wiest and Heidelberger (1953*b*).

In the present investigation of the role of soluble proteins in the elimination of carcinogens, an examination has been made of the animal serum collected at intervals after intravenous injection of benzpyrene colloid. A preliminary communication of this work has been made (Chalmers, 1953). The serum had a blue fluorescence the intensity of which appeared to be maximal after 1 to 1½ hours. Heidelberger and Weiss (1951) found that 1½ hours after intravenous injection of 0·45 mg. benzpyrene-5-C^{14} as colloid the plasma contained 0·22 per cent of the dose while carcass + bile and gall bladder contained 72 per cent, gastro-intestinal tract + contents 11·7 per cent, and carcass fat + tissue 9·6 per cent respectively. Thus the fluorescent constituent present in the serum at any one time represents only a small fraction of the injected hydrocarbon.

EXPERIMENTAL.

Solutions of hydrocarbons in serum.—An excess of the solid hydrocarbon was mixed with 0·2 to 2·0 ml. human, rat, fowl or mouse serum by grinding the hydrocarbon in the particular sample of serum with a glass rod and shaking at room temperature for about 30 minutes. Mixing was continued until the serum appeared to be saturated as indicated by the fluorescence of the solution in the ultra-violet beam. In the case of benzpyrene the supernatant serum gradually assumed a violet fluorescence which contrasted with the yellow fluorescence of the undissolved hydrocarbon. Excess hydrocarbon was then spun down and if sufficient serum was available it was filtered through Whatman No.1 filter paper.

Micro-electrophoresis of protein on filter paper.—The apparatus described by Flynn and De Mayo (1951) was used. Protein zones were stained with naphthalene black and lipid with Sudan 4 or Sudan black.

Fluorescence spectrum analysis.—Fluorescence spectra were recorded on a Hilger E3 spectrograph using Ilford HP3 hypersensitive panchromatic plates. The intensity of the fluorescence spectrum bands of known concentrations of benzpyrene was compared with that of the unknown substance in the same solvent photographed on the same plate, to give an estimate of the amount of benzpyrene present.

Total fatty acid was estimated by titration with alkali of the fatty acid from the hydrolysate of an alcohol-ether extract of the serum (Bloor, 1943).

Cholesterol was estimated by the Liebermann-Burchard reaction.

Wunderly and Pezold (1952) investigated, by an electrophoretic method, the solution of carcinogenic hydrocarbons in blood serum. Serum was applied to a strip of filter paper which had been dipped in a benzpyrene solution and afterwards dried. After electrophoresis the paper was cut in sections containing the various serum-protein zones each of which was eluted separately and the quantity of hydrocarbon in the eluate determined spectrophotometrically. They found

that α_2- and β-lipoproteins were mainly concerned with the process of solution and also that the percentage of benzpyrene which could be eluted increased with increasing lipid content of the serum.

RESULTS.

Human serum + benzpyrene in vitro.

Chromatography.—A spot of a solution of benzpyrene in human serum on filter paper was developed with water and another spot with saline until the solvent front had advanced about 15 cm. The paper was then dried, examined in ultra-violet, the fluorescent areas marked in pencil and the paper stained for protein. A similar pair of spots developed in the same way were stained for lipid. At the same time spots of control serum to which no benzpyrene had been added were examined as above. The fluorescence of the developed serum was associated with the protein staining area, the bulk of which had an R_f value of 0·6 to 0·9 although this highly mobile spot had a tail stretching towards the site of application. The lower fringe of the spot showed the maximum fluorescence and this portion also gave a lipid stain with Sudan black. Development with water, in which fluorescent material has a higher R_f value, gave better separation than with saline.

Control serum also gave a rapidly moving spot in which the protein and lipoprotein were concentrated. The natural pale blue fluorescence of the albumin was also observed. There was, in addition, a pale blue fluorescent zone corresponding in position to the lipoprotein fringe. The natural fluorescence however differed from the violet fluorescence of benzpyrene in intensity and colour.

The developed chromatogram of a series of spots of human serum + benzpyrene was dried and the lower 2–3 cm. of the paper, including the site of application of the drops, were detached. The upper portion of the chromatogram which was now free from any unchanged benzpyrene was cut into strip chromatograms of each drop and they were re-developed separately with various solvents—ether, petroleum ether, benzene, chloroform, acetone and alcohol. In the case of the non-polar solvents there was little change in the position of the fluorescent zone after development, but with the polar solvents there was a slight but definite movement. The association of benzpyrene with serum protein, however, is of a loose nature since shaking the serum with ether at room temperature results in a separation of the fluorescent material into the ether layer.

Essentially similar results were obtained with fluoranthene as with benzpyrene. Anthracene was also tested but the fluorescence was not sufficiently intense to distinguish the hydrocarbon fluorescence from that of the natural albumin.

Experiments designed to fractionate the serum proteins by chromatographic means were discontinued when it appeared that this could be more readily done by electrophoresis.

Zone electrophoresis.—The protein zones of a saturated solution of benzpyrene in human serum were separated by zone electrophoresis, 3 similar patterns being obtained on 3 strips of filter paper. One pattern was examined in ultra-violet while still wet with buffer and the two others after drying were stained for protein and lipid in the usual way. It was observed that the β-lipoprotein band showed a violet fluorescence and on elution with distilled water, benzpyrene was detected by fluorescence spectrum analysis. In one or two cases where the serum had a high lipid content a violet fluorescent α_1-lipoprotein band was also observed.

When, owing to the small amount of serum available, serum was used without filtering off the excess hydrocarbon, any undissolved material remained at the site of application. The albumin zone showed a natural pale blue fluorescence which contrasted with the violet fluorescence of benzpyrene associated with the β-lipoprotein. In cases where the difference in fluorescence was not marked confirmation was obtained by fluorescence spectrum analysis.

Concentration of benzpyrene.—The concentration of benzpyrene in human serum in the *in vitro* experiments varied with the lipid content of the serum, the highest value, 22 mg. per 100 ml., being recorded for serum from a patient with diabetes. Total fatty acid and cholesterol values were taken in two cases but in five others the cholesterol value was taken as an index of the total fatty acid content. Cholesterol values varied from 130 to 247 mg. per 100 ml., and benzpyrene from 4 to 20 mg. per 100 ml. The results indicated that the solubility of benzpyrene in human serum increases with increasing lipid concentration of the serum.

Human serum + hydrocarbons other than benzpyrene in vitro.

Zone electrophoresis.—The electrophoretic patterns of a number of aliquots of a sample of human serum to which aromatic hydrocarbons other than benzpyrene had been added *in vitro* were examined by zone electrophoresis. These preparatory experiments were made in order to determine the suitability of the hydrocarbon for *in vivo* experiments in which the behaviour of the compound could be compared with benzpyrene. The descending order of intensity of fluorescence of the β-lipoprotein band when examined under the ultra-violet lamp was as follows: fluoranthene, benzpyrene, methylcholanthrene, pyrene, cholanthrene, 3 : 4-benzphenanthrene, 2′ : 6- dimethyl 1 : 2-benzanthracene. In the case of fluoranthene only was a fluorescent α_1-lipoprotein band also visible.

Animal serum.—A solution of benzpyrene in rat serum was examined by zone electrophoresis as in the case of the *in vitro* experiments with human serum. After electrophoresis for 16–18 hours it was found that the rat lipoprotein was unstable under the conditions used, that the lipid staining area was spread over a relatively wide area (α- to γ-globulin bands), and that the fluorescence of the unstained pattern which was diffuse was not clearly visible. However, on carrying out the electrophoresis run for about one-half of the usual time, the fluorescence while still spreading over the α- to γ-globulin area was more clearly visible and corresponded with the liquid staining zone.

Mouse serum to which benzpyrene had been added *in vitro* gave very similar results to the rat experiments and again the lipoprotein appeared to be unstable under the conditions of electrophoresis.

In the case of fowl serum to which benzpyrene had been added *in vitro* evidence was obtained of a narrow lipid staining zone of low mobility similar to the β-lipoprotein of human serum. The violet fluorescence of the unstained β-lipoprotein band again as in the case of human serum corresponded with the lipoprotein zone.

In vivo *experiments.*

A fowl (weight 650 g.) was injected in the wing vein with 5 ml. of a benzpyrene colloid and killed 1½ hours later when it was known that there would be metabolised benzpyrene in the blood. The fluorescence of the unstained electrophoretic

pattern was examined and it was found that the albumin zone had a blue fluorescence not present in the serum of the animal before injection of the colloid.

Nine rats weighing 200–250 g. were injected in the tail vein each with 3–5 ml. benzpyrene colloid and the animals were killed at intervals between 15 minutes and 3½ hours after injection. Blood from the heart was allowed to collect in the thoracic cavity and the serum obtained was examined by zone electrophoresis. The albumin zone had a blue fluorescence not present in the serum of a control uninjected animal. The aqueous eluate of the fluorescent albumin zone of a number of strips was freeze-dried. A portion of this desiccate was reconstituted with water to give a 7–8 g. per cent solution and re-electrophoressed. A blue-fluorescent albumin zone was again formed on the paper strip. Spectrograph examination of the aqueous eluate of this zone and of the parent fluorescent albumin zone showed in both cases the spectrum of metabolised benzpyrene. It was noted however that the fluorescent substance in the freeze-dried preparation of the albumin zone was not appreciably soluble in ether but was readily soluble in hot alcohol. The fluorescence spectrum of the aqueous eluate of the fluorescent albumin zone or of the desiccate reconstituted with water showed bands with maxima at 4180 and 4440 Å and the spectrum was partly masked by the natural fluorescence of albumin. The alcoholic extract of the desiccate of the fluorescent albumin zone showed bands with maxima at 4150 and 4400 Å. In this case the bands were much sharper presumably owing to the absence of albumin, and the shift in the maxima may be due to the influence of the solvent.

Fluorescence was also associated with the globulin zones of the patterns in addition to that of the albumin zones, although on occasion this was masked owing to haemolysis of the serum and, as previously explained, by the diffuseness of the lipoprotein of rat serum under the conditions of electrophoresis. The globulin area of the pattern was separated, eluted with water, freeze-dried and reconstituted with water and in unhaemolysed samples the fluorescence spectrum of benzpyrene could be detected in the aqueous extracts. The spectrum bands were sharp and corresponded in position with those of a solution of benzpyrene in alcohol. Shaking with hot ethanol liberated the fluorescent material from a desiccate of the fluorescent globulin band and the fluorescence spectrum of the extract showed the benzpyrene bands.

DISCUSSION.

Benzpyrene is adsorbed by the lipoprotein of blood serum and its fluorescent metabolite is adsorbed by albumin. It is known that lipoprotein contains cholesterol and it was anticipated that benzpyrene, a non-polar substance, would be adsorbed by the blood stream in this way. On the other hand albumin binds substances that are relatively polar and this property may account for its affinity for the fluorescent benzpyrene metabolites which are more polar than the parent hydrocarbon. The slow but continuous absorption of benzpyrene or its metabolites into the blood stream after injection or skin painting could be effected in this way. The adsorbed compound then takes the physical property of the protein in solubility in aqueous medium and mobility on electrophoresis. The complexes of hydrocarbon or its metabolites with serum proteins are readily dissociated by shaking with hot alcohol. In this way they differ from benzpyrene

bound to skin protein, which when precipitated and dried withstands extraction with hot alcohol.

Other hydrocarbons, carcinogenic and non-carcinogenic, were also adsorbed *in vitro* by the lipoproteins of human serum and on electrophoresis migrated with the β-lipoprotein band.

Benzpyrene was found to be soluble in human serum to the extent of 40 to 220 μg/ml. depending on the lipid content of the serum. Wunderly and Pezold (1952) also found that the solubility of benzpyrene in serum depended on the lipid value. This value compares with Berenblum and Schoental's (1942) value of 40 μg/ml. for mouse serum *in vitro* and the same authors found values of 0·2 to 1 μg. benzpyrene in the total blood volume of a mouse at intervals of 1 hour to 5 days after intraperitoneal injection. Heidelberger and Weiss (1951) found in mouse serum 1½ hours after intravenous injection 0·22 per cent of 0·45 mg. benzpyrene-5-C^{14} injected, i.e. 1 μg. in the total blood volume.

Electrophoretic examination of the serum of rats fed with methylcholanthrene by gastric instillation has been made by Schultz, Jamison, Shay and Gruenstein (1954). They found an increase in the β-globulin protein component of rat serum and refer to the effect of lipid extraction which did not influence the β-globulin but produced instead a loss in the albumin area. Schultz *et al.* (1954) used boundary electrophoresis whilst the present results were obtained with zone electrophoresis, but it may be that as in the experiments described above the lipoprotein was spread over the globulin zone and hence lipid extraction would not produce an effect comparable with the effect on human serum, which has a sharp β-lipoprotein band.

Boundary electrophoretic examination has also been made by Cook, Griffin and Luck (1949) on the serum proteins of rats fed with carcinogenic azo dyes. Some of the azo dyes had little effect on the mobility of the serum proteins while *m'*-methyl-*p*-dimethylaminoazobenzene caused an increase in γ-globulin and a decrease in serum albumin. The authors state however that these changes may be non-specific since liver damage in humans can cause this type of protein change.

SUMMARY.

There is evidence that benzpyrene added *in vitro* to human or animal serum is associated with the lipoprotein fraction. This evidence was obtained by (1) chromatographic examination on filter paper in which the fluorescent compound was associated with lipid material having a high R_f value in water, and (2) zone electrophoresis in which the fluorescent compound migrated with the lipoprotein fraction of the serum.

There is also evidence that after intravenous injection of benzpyrene in rats and fowls the blood serum contains both unchanged and metabolised benzpyrene. Unchanged benzpyrene was associated with the globulin fraction of the serum and the metabolite with albumin.

The nature of the association of the hydrocarbon and its metabolite with protein has been discussed.

On zone electrophoresis human and fowl sera show the presence of a β-lipoprotein, but under the same conditions of electrophoresis, lipoprotein of rat and mouse sera appears to be unstable and forms a broad zone extending over the α- to γ-globulin bands.

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THE METABOLISM OF NEOPLASTIC TISSUES: SYNTHESIS OF CHOLESTEROL AND FATTY ACIDS FROM ACETATE BY TRANSPLANTED MOUSE TUMOURS *IN VITRO* AND *IN VIVO*.

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SEVERAL lines of experimentally induced ovarian tumours of the granulosa cell type were successively developed in the Biological and Endocrinological Department of our Institute (Mühlbock, 1952). In accordance with earlier observations (Furth and Butterworth, 1936; Li and Gardner, 1947), the transplanted tumours were found to produce oestrogens in many cases.

Chemical and biological analyses of the various tumours revealed distinct differences in their cholesterol and oestrogen content.

Although cholesterol is believed to be a common precursor for several steroid hormones (Lieberman and Teich, 1953), a rôle of cholesterol in the biosynthesis of the oestrogenic steroids is improbable (Heard *et al.*, 1954). Despite the fact that actual information regarding the path of oestrogen biogenesis is still lacking, a study of the metabolic pathways of the various tumours leading to lipid constituents should be of general interest, because oestrogens and cholesterol may still share precursors other than acetate. Work on the oestrogens is still in progress; the present communication serves to provide more information regarding the cholesterol and fatty acid synthesis in neoplastic tissues since relatively little information is at hand in this respect. The work already done by other investigators is mainly concerned with hepatomas (Baker and Greenberg, 1949; Olson, 1951; Zamecnik *et al.*, 1951; Medes, Thomas and Weinhouse, 1953) and in order to correlate the present data with the insight gained from the latter kind of tumour, some hepatomas were included in our experiments.

EXPERIMENTAL.

Animals and tumours.

Details of the tumours investigated are given in Table I. The tumours were all well established specimens, transplanted in the strains of mice indicated.

TABLE I.—*Details of the Mouse Tumours Studied.*

Case number and type of tumour.		Strain of mouse.	Transplanted after :
T5438	Ovarian tumours granulosa cell type	F1 (♀ C57 Black × ♂ DBA_f)	4 weeks
T5441		Ditto	4 „
T26567		„	8–12 „
T19957		F1 (♀ O20 × ♂ DBA_f)	16 „
T26567	Ovarian tumours sarcomatoid type	F1 (♀ C57 Black × ♂ DBA_f)	3 weeks
T24202		Ditto	3 „
T26473	Hepatomas	F1 (♀ O20 × ♂ DBA_f)	10 weeks
T15282		CBA	3–4 „

The spontaneous hepotomas studied arose in a high incidence in 2 year old F1 (♀ C57 Black × ♂ C_3He) (Mühlbock, unpublished communication). The hepatomas mentioned in Table I were transplanted from spontaneous tumours occurring in old mice of the CBA strain.

The ovarian tumours originated from (*a*) total body Röntgen irradiation (T5438 and T5441), (*b*) transplantation of the ovary in the spleen of a gonadectomized animal (T24202 and T19957) and (*c*) subcutaneous transplantation of the ovary (T26567) (Mühlbock, 1954).

The sarcomatoid transformation took place in the fourth transplantation of T24202 and in the second transplantation of T26567.

In the opinion of van Rijssel *et al.*, (1954) no real sarcomas were formed.

Substrates.

$BaC^{14}O_3$ was purchased from the Radiochemical Centre, Amersham, England. Sodium acetate-1-C^{14} was synthesized by carbonation of methyl magnesium bromide. After wet combustion (Slyke, Plazin and Weisiger, 1951) the specific activity amounted to $6{\cdot}1 \times 10^5$ counts/min. as an "infinitely thick" layer of $BaCO_3$ on a 1 square centimetre area.

Analytical pure reagents were used for the preparation of the Krebs Ringer phosphate buffer.

In vitro *experiments.*

For convenience, the full details of the incubation and subsequent procedures used in this and the following papers of this series are given here.

The animals were killed by decapitation and the tumours removed and immediately transferred to small beakers placed in crushed ice. Slices were cut in an apparatus consisting of rotating circular razor blades (de Flines, 1951). The slices were caught in aerated Krebs Ringer phosphate buffer at 0° C., blotted on filter-paper and weighed. Aliquots of 1 g. were transferred to 40 ml. incubation-flasks provided with a centre well for $C^{14}O_2$ collection. The vessels contained 5 ml. aerated Krebs Ringer phosphate buffer and 2·5 mg. sodium acetate-1–C^{14}.

Pure oxygen was passed through, then the flasks were stoppered with tight-fitting rubber serum bottle caps and gently shaken in a waterbath at 37° C. during four hours. At the end of the incubation period, 0·75 ml. of a 20 per cent CO_2-free NaOH solution was injected through the rubber caps into the centre well and about 0·2 ml. of 2N HCl into the medium (Kats and Chaikoff, 1954).

After an appropriate period, a layer of toluene was injected into the centre well and the caps were removed. The alkali was diluted with a concentrated NH_4Cl solution and transferred to a boiling solution of 10 per cent $BaCl_2$ under a shower of nitrogen. The resulting $BaCO_3$ was weighed, diluted with inactive $BaCO_3$ via the gas phase and assayed for radioactivity.

After homogenization of the tissue, it was washed once with 5 ml. of 10 per cent trichloroacetic acid (TCA) and twice with 5 per cent TCA. Nucleic acids were removed by extraction with 5 ml. of 5 per cent TCA during 15 minutes at 100° C.

The residue was extracted once with 5 ml. of 70 per cent ethanol, twice with 96 per cent ethanol and twice with absolute ethyl ether successively.

The residual protein was plated directly on planchets of 1 square centimetre

area as infinitely thick layers and the radioactivity determined. The alcohol and ether washings were combined, evaporated, the residue hydrolysed with alcoholic KOH and the resulting solution extracted 4 times with petroleum ether (b.p. 50–60° C). The petroleum ether extract was washed twice with a 1 per cent sodium acetate solution and once with distilled water. After evaporation of the solvent the crude cholesterol was dissolved in absolute ethanol and precipitated with digitonin. The cholesterol digitonide was filtered through a funnel according to Popjak (1950).

All counts reported are corrected if necessary to " infinitely thick " layers by means of a self-absorption curve constructed for cholesterol digitonide in the range of 0–25 mg. on 1 square centimetre area. The alcoholic alkaline solution, containing the potassium salts of fatty acids, was acidified and extracted 4 times with petroleum ether. This extract was washed twice with a solution of 1 per cent acetic acid and once with distilled water. With this procedure, an appreciable amount of short chain fatty acids soluble in water is inevitably lost. After evaporation of the petroleum ether the fatty acids were directly plated in disks of 1 square centimetre area on lenspaper as " infinitely thick " layers.

In vivo *experiments.*

Animals bearing 14–15-days-old tumour transplants of approximately uniform weight were selected, and injected intraperitoneally with 0·2 ml. saline containing 0·4 mg. radioacetate (3 μ C).

At fixed intervals 3 animals were decapitated and brain, liver and tumour removed. The tissues were hydrolyzed with alcoholic KOH. Cholesterol digitonide and fatty acids were isolated as described.

The *in vivo* experiments were repeated once, giving the same results as those reported.

Determinations.

Cholesterol was assayed by the colorimetric method of Bloor (1916, 1928); gravimetrical analysis of the digitonide yielded slightly smaller values. The latter were used for calculation of the data. The higher fatty acids were isolated as described and determined gravimetrically.

Determination of the liver *glycogen* as the sum of glucose and glycogen was based upon the method of Mendel, Kemp and Myers (1954).

Coenzyme A was assayed by the method of Handschumacher, Mueller and Strong (1951). A coenzyme A preparation purchased from Pabst Laboratory served as a reference. It contained approximately 75 per cent of the reduced form of the coenzyme, equivalent to about 300 Lipmann units per mg. by the method of Kaplan and Lipmann (1948).

Oestrogens.—Fresh tumour tissue was lyophilized immediately after removal from the animal and the dry material powdered in a mortar. Continuous extraction of this powder with chloroform for eight hours yielded an extract which was purified and solved in 1·2 ml. olive oil after the removal of the chloroform *in vacuo*. The oily solution was administered to ovariectomized mice in 3 subcutaneous injections over a period of 36 hours and the oestrogenic effect was measured by repeated vaginal smears.

Radioactivity measurements were made by standard methods. Counting was

done with an end-window Geiger Müller counter. In all cases sufficient counts were made in order to keep the probable error below 5 per cent.

Aliquots of fatty acids and cholesterol digitonide were combusted and the $BaCO_3$ was assayed for radioactivity in order to determine the appropriate conversion factors necessary for the calculations of the incorporation data.

RESULTS AND DISCUSSION.

In vitro *Experiments.*

A. *Transplanted tumours of the mouse ovary (granulosa cell and sarcomatoid type).*

Preliminary experiments showed that no radioactivity was detectable in the digitonin precipitable fraction obtained from ovarian tumour T 5438 after incubation of the tumour slices with acetate-1-C^{14} of rather low specific activity; a small but significant activity was observed in the cholesterol derived from Tumour 5441. However, experiments in which acetate-1-C^{14} of higher specific activity was used demonstrated that Tumour 5438 was capable of synthesizing cholesterol *in vitro*. The data are not reported here because they were obtained under conditions which were not always uniformly chosen. The results revealed a striking difference in the cholesterol content of the two ovarian tumours which were both of the granulosa cell type. The original difference observed in counts/min. of the respective cholesterol fractions was very probably due to the variation in cholesterol content, thereby causing a higher dilution of activity in Tumour 5438 than in Tumour 5441. Tumour 5438 was not further studied in detail. Other tumours of this kind were also found to contain varying amounts of cholesterol, as is shown in Table II.

TABLE II.—*Cholesterol and Fatty Acid Content of Transplanted Ovarian Tumours of the Mouse.*

Tumour.	Type.	Cholesterol (mg./g. wet weight of tissue).	Long chain fatty acids (mg./g. wet weight of tissue).
T 5438 (g)	Granulosa cell	6–10	—
T 5441 (g)	Ditto	4– 5	13
T19957 (g)	,,	10–15	13–15
T26567 (g)	,,	6– 7	18
T26567 (s)	Sarcomatoid	2·5	11
T24202 (s)	,,	2·6	16

Granulosa cell tumours are known to produce oestrogens (Mühlbock, 1952) and a hormonal effect could always be noted on the secondary sex organs of the host bearing the granulosa cell tumours mentioned in Table II. Among these, Tumour 19957 was outstanding by its high content of oestrogenic material which was readily extractable from the lyophilized tumour tissue. We were unsuccessful however in preparing extracts containing oestrogenic activity from the other tumours.

In repeatedly transplanted carcinomas certain changes in histological structure have long been known and are referred to as sarcomatous transformations. This transformation has also been observed in granulosa cell tumours of the ovary

(van Rijssel *et al.*, 1954) and is accompanied by a faster growth rate of the tumour transplants and a loss of oestrogenic activity.

In contrast to the rather high cholesterol content of the granulosa cell tumours, the sarcomatoid tumours were found to contain a much smaller amount of steroid. The fatty acid content did not show this drop.

TABLE III.—*Biosynthesis of Cholesterol and Fatty Acids from Acetate-1-C*^{14} *by Slices of Transplanted Granulosa Cell and Sarcomatoid Tumours of the Ovary (Mouse).*

Number of experiments.	Tumour.	Cholesterol. Counts/min.*	Cholesterol. mmoles acetate incorporated per g. wet weight of tissue. $\times 10^5$.	Fatty acids. Counts/min.*	Fatty acids. mmoles acetate incorporated per g. wet weight of tissue. $\times 10^5$.
14	T 5441(g)	1200± 540 (400–2400)	3·8 ±1·7 (1·3 –7·6)	2700±1580 (730–6100)	14·6± 8·5 (4·0–33·3)
6	T 19957(g)	152± 44 (64– 228)	1·1 ±0·32 (0·46–1·65)	992± 235 (640–1360)	5·8± 1·4 (3·7– 7·7)
3	T 26567(g)	268± 44 (180– 340)	1·1 ±0·18 (0·8 –1·4)	2426± 623 (1522–3346)	18± 4·5 (11·3–24·8)
6	T 26567(s)	2408± 840 (1428–4476)	4·2 ±1·5 (2·5 –7·8)	729± 362 (308–1482)	3·4± 1·7 (1·5– 6·7)
9	T 24202(s)	844± 496 (160–2656)	1·5 ±0·9 (0·3 –4·9)	600± 200 (261– 815)	4·0± 1·3 (1·7– 5·4)

* " Infinitely thick " layers of cholesterol digitonide and fatty acids were plated directly on 1 square centimetre area. The cholesterol data were obtained by multiplying the counts per minute of cholesterol digitonide by four.

The results of the experiments in which the cholestero- and lipogenesis† of these tumours have been studied are given in Table III. Although the granulosa cell (g) type tumours contain more cholesterol than the sarcomatoid (s) type, the rate of cholesterol synthesis on a whole does not differ markedly among the two groups.

On the other hand the (g) type tumours seem to possess a somewhat higher rate of fatty acid synthesis than the (s) type.

In discussing the individual (g) and (s) types, the tumours 26567 lend themselves to a fair comparison since they descend from the same tumour. In this case differences in metabolic activity are pronounced, showing a higher rate of cholesterogenesis and a lower rate of lipogenesis in the (s) type of the tumour.

Applying the results concerning the rates of synthesis and content of cholesterol to *in vivo* conditions the most plausible explanation seems that cholesterol catabolism is, in general, more extensive in the (s) types of the tumours, and especially so in Tumour 26567(s). Because of its high oestrogenic content Tumour 19957 was of special interest and particularly since the highest amount of cholesterol was found in this tumour. However, the rate of cholesterogenesis was definitely not the highest and lipogenesis was of the lowest order among the tumours of the (g) type.

† In order to facilitate description, the terms lipogenesis for fatty acid synthesis, and cholesterogenesis for cholesterol synthesis, have been used.

B. *Hepatomas* (transplanted, spontaneous).

It was found that several hepatomas of various origin may also vary in their synthetic properties.

Data on two transplanted and one spontaneous hepatoma are given in Table IV.

TABLE IV.—*Biosynthesis of Cholesterol and Fatty Acids* in vitro *from Acetate-1-C*^{14} *by Slices of Mouse Hepatomas.*

Tumour.	Cholesterol. mg./g. wet weight of tissue.	Cholesterol. mmoles acetate incorporated per g. wet weight of tissue. $\times 10^5$.	Fatty Acids. mg./g. wet weight of tissue.	Fatty Acids. mmoles acetate incorporated per g. wet weight of tissue. $\times 10^5$.
T 15282	3·7	35·1	25	17·5
		35·8		24·7
		33·1		—
T 26473	5	6·25	50	36·4
		3·75		15·0
		7·0		32·6
Spontaneous hepatoma	6	36·1	50	86·1
		44·7		105

Although Tumour 15282 was grossly contaminated with necrotic areas, which were impossible to remove, its rate of cholesterogenesis was of the same order as that of liver. At the end of the present experiments, this hepatoma did not "take" on further transplantation. An experiment with a tumour of the last generation at hand revealed a lowering of all synthetic capacities as compared with the three earlier experiments reported in Table IV. The transplanted Hepatoma 26473, which was practically free of necrosis, showed a low rate of cholesterol synthesis. Such a difference was not observed between the syntheses of fatty acids in both tumours. The cholestero- and lipogenesis were pronounced in the spontaneous hepatomas. For obvious reasons these data cannot be compared with the others; it should be noted that the livers of the hybrids, in which a high incidence of these spontaneous hepatomas was observed (Mühlbock, unpublished communication) contain a large amount of fatty material.

C. *Levels of coenzyme A in the tumours.*

Since acetate is being converted to acetyl coenzyme A, prior to its participation in metabolic reactions, the level of coenzyme A (CoA) might be a rate limiting

TABLE V.—*Coenzyme A Content of Some Mouse Tumours.*

Tumours.	Number of determinations.	Lipmann units per g. fresh tissue.
Of the ovary:		
T 5441(g)	8	20–30
T26567(s)	3	15–22
T24202(s)	5	10–15
Hepatomas:		
T15282	4	11–17
T26473	2	20–30
Spontaneous hepatoma	2	50–70

factor in the biosynthetical reactions studied in the present investigation. Therefore the content of CoA was determined in several of the tumours studied (Table V). The levels found in the transplanted mouse tumours were of the same order as those reported in the literature for a number of rat tumours (Strenght and Seibert, 1954 ; Higgins, Miller, Price and Strong, 1950).

In the group of the transplanted ovarian tumours (g and s type), the CoA content of the tumours appears to broadly parallel their synthetical capacities *in vitro*. On the other hand, a discrepancy in the CoA content and level of syntheses of the Tumours 5441 and 15282 was found. From this it was concluded that, in general, the content of CoA need not be the rate limiting factor in the biosynthetical reactions studied.

A less pronounced drop in the CoA content of the spontaneous as compared with the transplanted hepatomas, exists with respect to the normal liver. The livers of the tumour-bearing animals were also assayed. For the greater part no significant deviations from the values obtained for normal liver (100–150 Lipmann units per gram fresh tissue) were found except in some cases with animals bearing the Tumour 5441 during the longest tolerable period (up to forty-five days). A considerable drop in CoA content was repeatedly noted then.

D. *Livers of normal and tumour-bearing* F_1 (♀ *C57 Black* × ♂ *DBA*$_f$)† *mice.*

Mice bearing transplants of a granulosa cell tumour of the ovary develop an increased blood volume and an enlarged and spongy liver (Mühlbock, 1952). The normal liver was found to contain 6·7 per cent glycogen on the average, while its counterpart in the T5441-bearing animals contained a smaller percentage, varying from 2·3–4·4 per cent. The condition and the metabolic activity of the liver of the intact tumour-bearing animal may be of interest since it can be assumed that only part of the cholesterol and fatty acids (Medes, Thomas and Weinhouse, 1953. *cf.* E) is synthesized in the tumour, whereas the remaining is derived from plasma cholesterol and fatty acids which originate from the liver. In this connection the synthetic capacities of the livers of mice bearing T5441-transplants were compared with the livers of the corresponding normal mice (Table VI). Since the glycogen content has been shown to be directly related to the amount of acetate carbon converted to fatty acid by liver slices (Haugaard and Stadie, 1952), a difference between the incorporation of tracer could be expected in these

TABLE VI.—*Biosynthesis of Cholesterol and Fatty Acids* in vitro *from Acetate-1-*C^{14} *by Liver Slices of Normal and T5441-bearing BDz Mice.*

mmoles acetate incorporated per g. fresh tissue. × 10^5.			
Normal liver.		Liver of tumour-bearers.	
Cholesterol.	Fatty acids.	Cholesterol.	Fatty acids.
30·7	40·6	12·2	24·3
61·4	65·7	30·0	57·2
80·3	51·3	27·9	28·6
24·3	47·6	39·2	22·4
		77·5	235·0
Mg./g. wet weight.	Mg./g. wet weight.	Mg./g. wet weight.	Mg./g. wet weight.
2·8	11	3	16

† abbreviated as BDz.

experiments. Except for one experiment in which an unusually high incorporation was found, the average incorporation of acetate into fatty acids in the remaining five experiments with livers of tumour-bearing animals appears to be on a lower level than the corresponding value of normal liver. Almost the same does relate to the synthesis of cholesterol.

Further studies are needed however to establish a strict relationship between tumour weight, weight, and glycogen content of the livers, on one hand, and the lipogenesis of the liver on the other.

E. In vivo *experiments.*

The labelling of cholesterol and fatty acids in normal and neoplastic tissue of F_1 (♀ C57 Black × ♂ DBA_f) mice bearing the transplanted ovarian tumour T5441

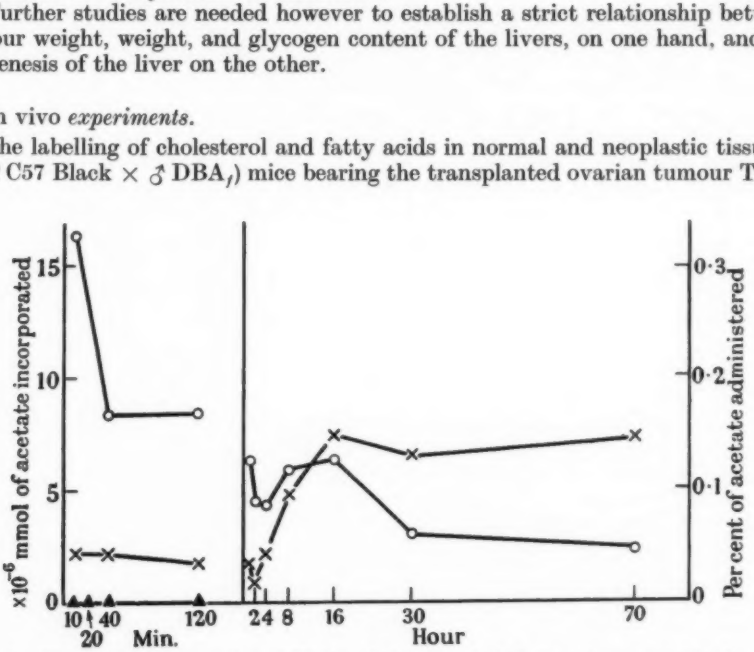

FIG. 1.—The *in vivo* incorporation of C^{14} into cholesterol of whole liver, tumour and brain of male BDz mice bearing the transplanted ovarian tumour 5441 after an intraperitoneal injection of 0·4 mg. acetate-1-C^{14} (3 μC). Total wet weight: liver 1·5 g.; tumour 3·5 g. Total cholesterol content: liver 4·2 mg.; tumour 16 mg. ○——○ liver; ×——× tumour; ▲——▲ brain.

and of CBA mice bearing the transplanted hepatoma T15282 was studied after an intraperitoneal injection of 3 μC sodium acetate-1-C^{14}. In these experiments the incorporation of tracer into cholesterol and fatty acids of the liver, brain and the tumour was followed over both a 2 and a 70-hour period.

Typical results obtained with animals bearing the granulosa cell tumour of the ovary are presented in Fig. 1 and 2 and those with mice bearing the hepatoma in Fig. 3 and 4. In all figures the total amount of acetate incorporated into the respective constituents of the whole organ is plotted against time. The curves thus obtained illustrate a rapid turnover of liver cholesterol and fatty acids in tumour-bearing animals, studied earlier in normal animals by Hevesy, Ruyssen and Beeckmans (1951), Beeckmans, Casier and Hevesy (1951), and Hutchens, van Bruggen and West (1954).

It was not possible to detect any measurable radioactivity in the brain cholesterol under the conditions of the present experiments. As it was felt that

the presence of the tumour could possibly drain off radioactive precursors normally reaching the brain, an identical experiment was performed with normal mice of identical genetic constitution. In this experiment also the brain cholesterol was found to contain no radioactivity. Concomitantly with the decrease in total radioactivity of the liver cholesterol, the corresponding activity shown by the cholesterol of the ovarian tumour slowly increased with time, reaching a maximum at about 16 hours. The total activity incorporated into the cholesterol of the tumour tissue (3·5 g. on the average) surpassed that of liver at about 12 hours after administration of the radioacetate and remained approximately constant from 16 hours until the end of the experiment. These data reflect an active cholesterol catabolism in a non-growing tissue, the liver, and little if any catabolic activity in the growing tissue of the tumour T5441. Thus, this tumour can be considered as behaving like a cholesterol " trap ".

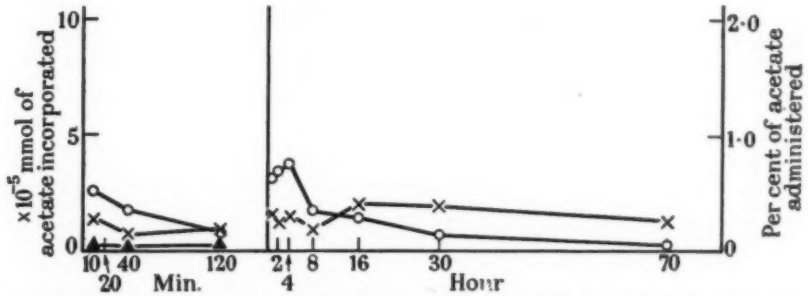

FIG. 2.—The *in vivo* incorporation of C^{14} into long chain fatty acids of whole liver, tumour and brain of male BDz mice bearing the transplanted ovarian tumour 5441 after an intraperitoneal injection of 0·4 mg. acetate-1-C^{14} (3 μC). Total wet weight: liver 1·5 g.; tumour 3·5 g. Total content of fatty acids: liver 17 mg.; tumour 20 mg.; brain 11 mg. ○——○ liver; ×——× tumour; ▲——▲ brain.

The time course of cholesterol labelling in liver and tumour demonstrates that C^{14} is transferred from the liver to the tumour area and is stored there in cholesterol. Synthesis *de novo* in the tumour depends on the supply of acetate to the tumour. Although no data are available on the time sequence of acetate disappearance from blood after intraperitoneal injection into tumour-bearing mice, it seems justified, in view of the experiments with rats by Busch (1953) and Busch and Baltrush (1954), to assume that within a very short time (10 minutes) the amount of radioacetate remaining in the blood forms but a very small percentage of the original. To what degree the two processes resulting in the cholesterol labelling of the tumour are operating remains undecided but it appears from what has been said that *total* synthesis will be of importance in the very beginning of the experiment, whereas transfer from the liver will dominate in the later stages. As a consequence the blood supply has its definite bearing on both processes. The ovarian tumour is nearly free of necrosis and the blood supply is rather well organized. This does not hold true for the hepatoma. Notwithstanding this, the total incorporation of tracer into hepatoma cholesterol after 10 minutes is much higher than that into the cholesterol of the ovarian tumour, even though the latter surpasses the former more than twice in weight.

The data of these *in vivo* experiments seem to confirm the *in vitro* results, i.e. the much higher rate of cholesterol synthesis in the hepatoma as compared with that of the ovarian tumour. The fact that cholesterol labelling of the hepatoma

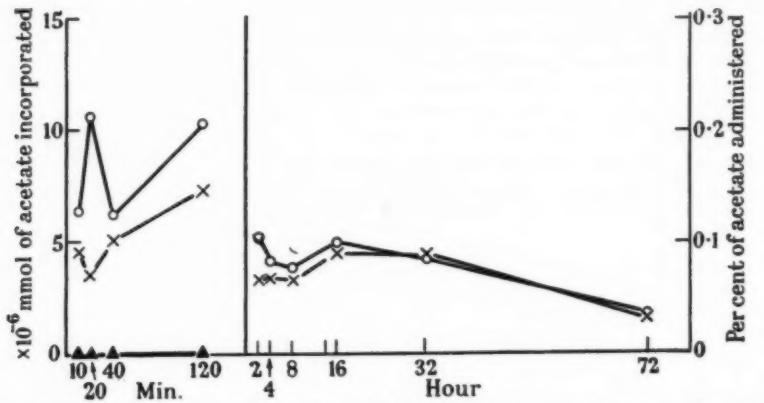

FIG. 3.—The *in vivo* incorporation of C^{14} into cholesterol of whole liver, tumour and brain of female CBA mice bearing the transplanted hepatoma 15282 after an intraperitoneal injection of 0·4 mg. acetate-1-C^{14} (3 μC). Total wet weight: liver 1·5 g.; tumour 1·5 g. Total cholesterol content: liver 4·1 mg.; tumour 5·6 mg. ○——○ liver; ×——× tumour; ▲——▲ brain.

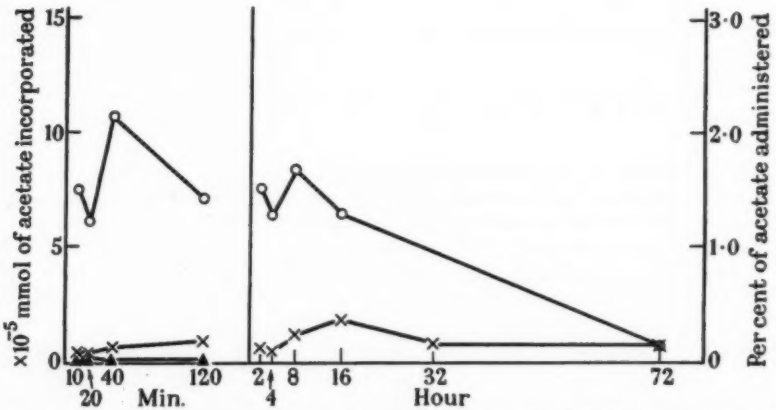

FIG. 4.—The *in vivo* incorporation of C^{14} into long chain fatty acids of whole liver, tumour and brain of female CBA mice bearing the transplanted hepatoma 15282 after an intraperitoneal injection of 0·4 mg. acetate-1-C^{14} (3 μC). Total wet weight: liver 1·5 g.; tumour 1·5 g. Total content of fatty acids: liver 36 mg.; tumour 38 mg.; brain 11 mg. ○——○ liver; ×——× tumour; ▲——▲ brain.

never exceeded that of the liver must be partially due to the low mass of the tumours (1·5 g. on the average). In this respect liver and tumour may be compared since they have approximately the same weight. The total incorporation of radio-

activity in the cholesterol fraction of this tumour was much like that of the liver, as can be seen in Fig. 3.

The results of the total incorporation of C^{14} into the fatty acid fractions of both tumours and the livers of the corresponding animals are illustrated by Fig. 2 and 4. It appears from this, that the livers of hepatoma-bearing mice incorporate much more of the administered tracer into their fatty acid components than the livers of the ovarian tumour-bearing animals. Apart from the strain difference it should be borne in mind that livers of granulosa cell tumour-bearing animals show an enlarged and spongy appearance. This may have its bearing on the difference in lipogenesis observed.

The total amount of acetate incorporated into the fatty acids of both tumours does not differ markedly.

The authors wish to acknowledge their indebtedness to Dr. O. Mühlbock, Dr. Th. G. van Rijssel and Dra. R. van Nie for many helpful discussions regarding the biological problems inherent in the present investigation and for making available the experimental animals and tumours.

SUMMARY.

1. The biosynthesis of cholesterol and long-chain fatty acids in surviving tissue slices of several transplanted mouse ovarian tumours of the granulosa cell type, and the sarcomatoid type was studied with acetate-1-C^{14}.

2. Granulosa cell tumours of the ovary contained more cholesterol than the sarcomatoid type of these tumours. Among the former group, the tumour with the highest oestrogen titre was found to contain the highest amount of cholesterol but not the highest rate of cholesterogenesis. The granulosa cell and sarcomatoid tumour which descended from the same tumour showed differences in the rates of their respective metabolic reactions leading to cholesterol and fatty acids.

3. Although the coenzyme A content of the tumours was found to parallel their synthetic capacities broadly, the level of CoA need not be the rate limiting factor in the biosynthetic processes studied.

4. The livers of mice bearing a granulosa cell tumour were studied, but no clear-cut conclusions as compared with normal liver could be made.

5. Including the results obtained from identical experiments with three hepatomas, it can be stated in general that a distinct rate of synthesis *in vitro* was not found to be an exclusive property of all tumours belonging to one of the three groups studied.

6. The time course of labelling of the cholesterol and fatty acids in the liver, brain and tumour of intact mice bearing a granulosa cell tumour or hepatoma, was followed after administration of a single dose of acetate-1-C^{14}. The *in vivo* data of the hepatoma cholesterol resembled those of the liver, whereas little cholesterol catabolism was found to occur in the granulosa cell tumour. Hepatic lipogenesis differed markedly in the two cases.

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THE METABOLISM OF NEOPLASTIC TISSUES: THE RELATION OF CARBOHYDRATE UTILIZATION TO CHOLESTEROL AND FATTY ACID SYNTHESIS IN TUMOUR TISSUE SLICES.

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AN interrelation between carbohydrate utilization and fatty acid synthesis is firmly established (Bloch, 1952; Artom, 1953). Liver slices from fasted rats showed an impairment of lipogenesis which could be repaired by previous administration of glucose to the animals or by the addition of glucose to the incubation mixture (Lyon, Masri and Chaikoff, 1952; Masri, Lyon and Chaikoff, 1952). Fatty acid synthesis from acetate has been shown to be directly related to the glycogen content of the liver (Haugaard and Stadie, 1952). In studying this interrelation between carbohydrate and fat metabolism, the liver does not appear to be the most suitable tissue because of its high glycogen content. Although fasting can lower this, the early glucose phosphorylation is impaired by this procedure (Wyshak and Chaikoff, 1953). For this reason, Hirsch, Baruch and Chaikoff (1954) studied the lactating mammary gland, which stores practically no glycogen.

Since many neoplastic tissues do not store glycogen either, and show a high rate of aerobic glycolysis and a low rate of oxidation on addition of glucose, the interrelation between carbohydrate metabolism and synthesis of cholesterol and fatty acids was studied using surviving tissue slices of a number of transplanted mouse tumours.

EXPERIMENTAL.

The experimental conditions are described in detail in the preceding paper (Emmelot and Bosch, 1955). In order to investigate whether the radioactivity of the proteins was not simply due to an adsorption phenomenon a sample of protein was subjected to ninhydrine treatment before and after acid hydrolysis (Zamecnik *et al.*, 1948). The ratio of the specific activities of the $C^{14}O_2$ recovered was 1 : 27, indicating a considerable preponderance of protein bound amino acids.

RESULTS AND DISCUSSION.

In the present investigation the four main end-products of the acetate metabolism—carbon dioxide, protein bound amino acids, cholesterol and long-chain fatty acids—were isolated and assayed for radioactivity, after incubation of surviving tissue slices of some mouse tumours with acetate-1-C^{14}. The experiments were performed in the presence and in the absence of unlabelled fructose or glucose (150 μM) in the incubation medium.

The following four transplanted tumours were studied : ovarian tumours of the granulosa cell type (T5441), ovarian tumours of the sarcomatoid type (T24202 and T26567), and hepatomas (T15282).

Tables I to IV illustrate the results obtained with each of the four tumours separately.

TABLE I.—*Effect of Unlabelled Glucose or Fructose on the Incorporation of C^{14} from Acetate-1-C^{14} into CO_2, Protein, Cholesterol and Fatty Acids by Surviving Tissue Slices of the Transplanted Mouse Ovarian Tumour T24202 of the Sarcomatoid Type.*

For each experiment tumour slices were pooled and 1 g. of slices were incubated in 5 ml. Krebs Ringer phosphate buffer containing 2·5 mg. sodium acetate-1-C^{14} with and without unlabelled glucose or fructose added in a final concentration of 150 μM.
Duplicate flasks were incubated and each figure is the average of two separate determinations.
The data on CO_2, cholesterol and fatty acids are expressed per 1 g. of fresh tumour tissue. The counts/min. of the proteins are given as infinitely thick layers on 1 square centimetre area.

Carbon source.	CO_2. mmoles acetate converted × 10^3.	Protein. counts/min.	Cholesterol. mmoles acetate incorporated × 10^5.	Fatty acids. mmoles acetate incorporated × 10^5.
A	1·96	550	1·80	3·88
A + F	2·55	735	3·51	10·6
A + G	3·57	953	3·70	34·0
A	1·75	239	1·76	5·24
A + F	1·86	349	2·87	14.6
A + G	1·50	200	2·50	21·2
A	2·68	—	—	4·60
A + F	2·67	—	—	18·9
A + G	2·70	—	—	33·4

Abbreviations : A = acetate-1-C^{14}, G = glucose and F = fructose.

TABLE II.—*Effect of Unlabelled Glucose or Fructose on the Incorporation of C^{14} from Acetate-1-C^{14} into CO_2, Protein, Cholesterol and Fatty Acids by Surviving Tissue Slices of the Transplanted Mouse Ovarian Tumour T26567 of the Sarcomatoid Type.*

For conditions of experiment and abbreviations see Table I.

Carbon source.	CO_2. mmoles acetate converted. × 10^3.	Protein. counts/min.	Cholesterol. mmoles acetate incorporated. × 10^5.	Fatty acids. mmoles acetate incorporated. × 10^5.
A	1·53	410	3·26	2·85
A + F	1·48	470	4·12	5·52
A + G	1·92	605	6·13	13·5
A	1·61	366	6·35	5·75
A + F	1·63	355	10·1	20·1
A + G	1·50	264	4·49	20·0

TABLE III.—*Effect of Unlabelled Glucose or Fructose on the Incorporation of C^{14} from Acetate-1-C^{14} into CO_2, Protein, Cholesterol and Fatty Acids by Surviving Tissue Slices of the Transplanted Mouse Ovarian Tumour T5441 of the Granulosa Cell Type.*

For conditions of experiment and abbreviations see Table I.

Carbon source.	CO_2. mmoles acetate converted. $\times 10^3$.	Protein. counts/min.	Cholesterol. mmoles acetate incorporated. $\times 10^5$.	Fatty acids. mmoles acetate incorporated. $\times 10^5$.
A	3·95	1215	6·62	17·8
A + F	3·93	943	8·25	50·7
A + G	4·71	660	7·42	56·9
A	2·23	602	5·97	6·87
A + F	2·72	681	9·0	17·9
A + G	2·18	500	9·0	21·2
A	2·63	487	2·43	6·03
A + F	2·75	431	4·01	19·9
A + G	2·24	414	4·45	24·7

TABLE IV.—*Effect of Unlabelled Glucose or Fructose on the Incorporation of C^{14} from Acetate-1-C^{14} into CO_2, Protein, Cholesterol and Fatty Acids by Surviving Tissue Slices of the Transplanted Mouse Hepatoma T15282.*

For conditions of experiment and abbreviations see Table I.

Carbon source.	CO_2. mmoles acetate converted. $\times 10^3$.	Protein. counts/min.	Cholesterol. mmoles acetate incorporated. $\times 10^5$.	Fatty acids. mmoles acetate incorporated. $\times 10^5$.
A	6·42	1973	35·4	21·0
A + F	6·23	1802	52·7	55·0
A + G	6·71	1816	66·0	62·1
A*	4·17	1072	15·7	2·30
A + G	4·11	1032	46·0	24·3

* See text.

The most prominent of the data are those concerning fatty acid synthesis. A considerable stimulation of lipogenesis, varying from three to tenfold, was found after the addition of glucose with all four tumours studied.

A distinct level of stimulation could not be attributed to each one of the four tumours in particular, though the highest stimulation was found with T24202.

Experiments in which fructose was added were run simultaneously; marked stimulation was found in them also, but in general on a lower level as compared with glucose.

The data concerning the influence of glucose utilization on cholesterol biosynthesis were somewhat inconsistent. In 9 duplicate experiments, with glucose included in the incubation mixture, a stimulation of cholesterol biosynthesis of more than 20 per cent was found in 7 cases.

Not reported here are three earlier experiments with T5441 incubated in a Krebs Ringer bicarbonate buffer in which glucose was found without effect on

cholesterogenesis. Addition of fructose, on the other hand, has always led to an increased cholesterogenesis.

In those cases where a stimulation of cholesterol synthesis was found it was never of the order of that seen in lipogenesis. Thus, it can be concluded that lipogenesis is more dependent upon carbohydrate utilization than cholesterogenesis in the tumours studied.

Besides the pathways leading to fatty acid and cholesterol, the two-carbon fragments derived from acetate-1-C^{14} undergo oxidative degradation via the Krebs cycle yielding carbon dioxide. As a consequence of the latter reaction sequence, amino acid precursors (a.o. α-ketoglutaric acid) are steadily being formed, leading to the synthesis of protein. When using radioacetate as the sole carbon source a similarity between the incorporation of tracer into carbon dioxide and protein can thus be expected. Such a parallel was found to exist roughly, as can be seen from the tables. It was reported in the previous paper (Emmelot and Bosch, 1955) that the hepatoma T15282 did not "take" on further transplantation. Experiments with a tumour of the last generation at hand revealed a lowering of all synthetic capacities as compared with earlier data. In this case glucose had a very significant stimulatory effect on both cholestero- and lipogenesis. As can be seen from the last experiment reported in Table IV, cholesterol synthesis and fatty acid synthesis were increased 300 and 1000 per cent respectively; these were the highest stimulations found in the present work.

Another fact worth mentioning was the high specific activity of the respiratory carbon dioxide in both experiments with the hepatomas, as compared with other tumours. Thus, in this particular hepatoma, the oxidative pathway of acetate metabolism relative to endogenous C_2 oxidation is more pronounced than in the other three tumours, finding its equivalence in the labelling of the cholesterol and the protein of the hepatoma, but not to such extent in that of the fatty acids.

Neither the conversion of radioacetate to carbon dioxide nor its incorporation into protein, seems to be affected in a regular way by the simultaneous utilization of carbohydrate by the tumours. Although the stimulation of fatty acid synthesis by glucose addition to the tumour slices is of a lower magnitude than found by Hirsch, Baruch and Chaikoff (1954) studying the same effect in the rat mammary gland, the present data on the conversion of acetate-1-C^{14} to carbon dioxide are at variance with theirs.

Hirsch, Baruch and Chaikoff (1954) found a significant lowering of the amount of acetate oxidized after addition of glucose, and concluded from this that the oxidative utilization of glucose shifted the two-carbon fragments, derived from acetate, from an oxidative path to one involving lipogenesis.

The present data do not allow one to draw this conclusion; in fact, the stimulation in conversion of C^{14} of acetate-1-C^{14} to fatty acids, induced by carbohydrate, was never accompanied by a significant fall in the $C^{14}O_2$ recoveries. On the contrary an enhancement of acetate oxidation concomittant with the increase in lipogenesis was sometimes noted.

The differences between these findings may be due to the kind of tissues studied. Many normal tissues respond to the addition of carbohydrate by an increase in oxidation, whereas the tumour does so by glycolysis. Hence a shift of the two-carbon-C^{14} intermediates to synthesis, due to an increase in glucose oxidation, need not be the actual course of an enhanced fatty acid synthesis and especially not in tumour tissue. Since only a small quantity of the C2-intermediates from

unlabelled glucose reaches the Krebs cycle, because of the formation of lactic acid in the tumour tissue, it is evident that the stimulation which occurs must be due to glycolytic reactions. Although much work has already been done concerning the mechanism of the stimulatory action of glucose on fatty acid synthesis in normal tissues (Bloch, 1952), the exact cause of the effect is still obscure. The most plausible explanations are those which regard the formation of a metabolic " active form " of glycerol, the generation of adenosine triphosphate or both as determining the effect (Bloch, 1952). The first takes into account the formation of fat, since the absence of glycerol may be the rate limiting factor and the second envisages the energy requirements of the synthetic reactions concerned. Due to the active glycolysis encountered in the tumour tissues, both these substances can be generated in this reaction sequence and may be responsible for the stimulation of the lipid synthesis observed.

SUMMARY.

1. The incorporation of C^{14} of acetate-1-C^{14} in carbon dioxide, protein, cholesterol, and long-chain fatty acids by surviving tissue slices from a number of mouse tumour transplants has been studied in the presence and the absence of unlabelled fructose and glucose in the incubation mixture.

2. A marked stimulation of fatty acid synthesis was found after addition of glucose and somewhat less after addition of fructose.

3. Cholesterol synthesis was also found to be stimulated but to a smaller degree than fatty acid synthesis.

4. The stimulation in conversion of C^{14} of acetate-1-C^{14} to fatty acids and cholesterol, induced by the carbohydrates was never accompanied by a significant fall in the $C^{14}O_2$ recoveries.

5. It is concluded that the enhancement of fatty acid synthesis is directly related to the active glycolysis of the tumour tissues.

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THE METABOLISM OF NEOPLASTIC TISSUES: THE RELATIVE RATES OF ACETATE AND PYRUVATE UTILIZATION BY SURVIVING TISSUE SLICES OF MOUSE TUMOURS.

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IN the biosynthesis of both fatty acids and cholesterol the two-carbon fragment plays a rôle of importance. Earlier studies of these reactions in slices of tumour tissues to which acetate-1-C^{14} was added as a substrate did not reveal significant features in the metabolic rates common to each of the tumours belonging to one of three different groups studied (Emmelot and Bosch, 1955*a*, 1955*b*). In this connection pyruvate was of importance, since this compound might also serve as a precursor of the two-carbon fragment. In contrast with normal tissue, only very little information is available regarding the rôle of pyruvate in the synthetic processes of neoplastic tissue (Zamecnik *et al.*, 1951 ; Olson, 1951). The present communication deals with a comparison between the relative rates of the main metabolic reaction sequences in which acetate and pyruvate are engaged, using the three tumour groups studied earlier.

EXPERIMENTAL.

Sodium pyruvate-2-C^{14} was purchased from the Radiochemical Centre, Amersham, England. The radiopyruvate was diluted to the desired activity and an amount equivalent to the acetate-1-C^{14} was used, i.e., 1·875 mg. sodium-acetate ($6{\cdot}1 \times 10^5$ cpm as $BaCO_3$ as an " infinitely thick " layer on 1 square centimetre area) and 2·5 mg. sodium pyruvate ($2{\cdot}29 \times 10^5$ cpm under identical conditions, equivalent to $3{\cdot}435 \times 10^5$ cpm expressed as acetate-1-C^{14}). The counting data of the proteins following pyruvate incubation were calculated from the observed counts by multiplying with a factor 1·78 $\left(=\frac{6100}{3435}\right)$. All other data are expressed as mmoles of acetate incorporated or converted to CO_2 per 1 g. of fresh tissue. Pooled slices from one or more tumours were divided in two equal parts of 750 mg. each, and incubated separately in 3·75 ml. Krebs Ringer phosphate buffer supplemented with the radioactive substrates respectively. Further details are described in previous papers (Emmelot and Bosch, 1955*a*, 1955*b*).

RESULTS.

The incorporation of equivalent amounts of acetate-1-C^{14} and pyruvate-2-C^{14} into the carbon dioxide, protein-bound amino acids, long-chain fatty acids,

TABLE I.—*The* in vitro *Incorporation of Tracer from Acetate-1-C^{14} and Pyruvate-2-C^{14} into Respiratory Carbon Dioxide, Proteins, Cholesterol and Long-chain Fatty Acids by Transplanted Ovarian Tumours of the Granulosa Cell Type.*

In each experiment tumour slices from one or more animals were pooled and 750 mg. slices were incubated with 3·75 ml. Krebs Ringer phosphate buffer containing either 1·875 mg. acetate-1-C^{14} or 2·5 mg. pyruvate-2-C^{14} per flask. The data on CO_2, cholesterol and fatty acids are expressed per 1 g.of fresh tumour tissue. The counts/min. of the proteins are given as " infinitely thick " layers on 1 square centimetre area.

Animal number.	Tumour.	Substrate.	CO_2. mmoles of substrate converted. $\times 10^3$.	Proteins. counts/min.	Cholesterol. mmoles of substrate incorporated. $\times 10^5$.	Fatty acids. mmoles of substrate incorporated. $\times 10^5$.
566	T19957	acetate-1-C^{14}	1·63	217	1·00	3·70
566	„	pyruvate-2-C^{14}	7·57	3144	1·33	5·15
567	T19957	acetate-1-C^{14}	2·47	274	1·58	4·87
567	„	pyruvate-2-C^{14}	9·32	1374	1·91	47·0
568	T19957	acetate-1-C^{14}	1·40	247	0·83	4·55
568	„	pyruvate-2-C^{14}	7·23	1745	1·14	21·3
656+657	T 5441	acetate-1-C^{14}	2·28	418	1·50	10·3
656+657	„	pyruvate-2-C^{14}	4·19	956	1·05	17·5
658+660	T 5441	acetate-1-C^{14}	2·57	411	1·87	7·35
658+660	„	pyruvate-2-C^{14}	4·52	3050	3·33	24·1
661+662	T 5441	acetate-1-C^{14}	1·60	545	1·25	10·2
661+662	„	pyruvate-2-C^{14}	10·17	4710	3·22	26·8

TABLE II.—*The* in vitro *Incorporation of Tracer from Acetate-1-C^{14} and Pyruvate-2-C^{14} into Respiratory Carbon Dioxide, Proteins, Cholesterol and Long-chain Fatty Acids by Transplanted Ovarian Tumours of the Sarcomatoid Type.*

For conditions of experiment see Table I.

Animal number.	Tumour.	Substrate.	CO_2. mmoles of substrate converted. $\times 10^3$.	Proteins. counts/min.	Cholesterol. mmoles of substrate incorporated. $\times 10^5$.	Fatty acids. mmoles of substrate incorporated $\times 10^5$.
664+665	T24202	acetate-1-C^{14}	1·18	261	0·95	2·27
664+665	„	pyruvate-2-C^{14}	8·05	2593	19·6	11·3
666+667	T24202	acetate-1-C^{14}	0·97	242	1·44	—
666+667	„	pyruvate-2-C^{14}	6·21	2380	14·2	43·8
668+669	T24202	acetate-1-C^{14}	0·20	103	0·29	4·93
668+669	„	pyruvate-2-C^{14}	5·08	2037	7·63	13·4
768+769	T26567	acetate-1-C^{14}	0·83	227	3·35	1·44
768+769	„	pyruvate-2-C^{14}	4·57	1660	11·9	4·97
770+766+765	T26567	acetate-1-C^{14}	0·57	155	2·47	1·54
770+766+765	„	pyruvate-2-C^{14}	5·59	2065	19·7	8·77

and the digitonin precipitable steroid fraction was determined in tumour slices of four transplanted ovarian tumours, one transplanted and one spontaneous hepatoma. Two ovarian tumours were of the granulosa cell type and the other two of the sarcomatoid type.

The results of the experiments are summarized in Tables I, II and III. In all the tumours studied pyruvate appears to be more readily oxidized via the Krebs cycle than acetate, since the total amount of C^{14} present in the carbon dioxide following incubation with pyruvate-2-C^{14} always exceeded the total activity contained in carbon dioxide when the equivalent amount of acetate-1-C^{14} was used.

TABLE III.—*The* in vitro *Incorporation of Tracer from Acetate-1-C^{14} and Pyruvate-2-C^{14} into Respiratory Carbon Dioxide, Proteins, Cholesterol and Long-chain Fatty Acids by Transplanted and Spontaneous Hepatomas.*

For conditions of experiment see Table I.

Animal number.	Tumour.	Substrate.	CO_2. mmoles of substrate converted. $\times 10^3$.	Proteins. Counts/mins.	Cholesterol. mmoles of substrate incorporated. $\times 10^5$.	Fatty acids. mmoles of substrate incorporated. $\times 10^5$.
523+524	T26473	acetate-1-C^{14}	1·40	121	6·25	29·0
523+524	„	pyruvate-2-C^{14}	3·32	418	4·69	6·13
533+529	T26473	acetate-1-C^{14}	1·40	156	6·85	26·2
533+529	„	pyruvate-2-C^{14}	4·08	419	6·67	7·97
525	T26473	acetate-1-C^{14}	0·39	111	3·75	11·5
525	„	pyruvate-2-C^{14}	1·63	444	5·72	14·1
4000–4006	spontaneous hepatoma	acetate-1-C^{14}	2·40	385	36·1	86·1
4000–4006	Ditto	pyruvate-2-C^{14}	4·25	624	33·3	82·0
4000–4006	„	acetate-1-C^{14}	2·07	336	44·7	105·4
4000–4006	„	pyruvate-2-C^{14}	2·95	915	39·8	102·5

The sarcomatoid types oxidize pyruvate to a much greater extent than they do acetate. The least prominent difference between the two substrates, in this respect, exists in the case of the two hepatomas. The incorporation of tracer from pyruvate into tumour proteins also exceeds that from acetate in all of the six tumours investigated. Again in the hepatomas the preference of pyruvate utilization for protein synthesis seems less marked than in the other tumours. The differences which exist between the hepatomas, on one hand, and the remaining tumours on the other, are most clearly demonstrated by the data representing the lipogenesis.

In both the transplanted and the spontaneous hepatomas acetate incorporation into fatty acids equals or even surpasses pyruvate incorporation, whereas the opposite is true for the other four tumours.

As a whole, radioacetate is slightly superior to radiopyruvate in the extent to which the C^{14} of the C_2 fragment is incorporated into the hepatoma cholesterol. Although the absolute rates of pyruvate and acetate utilization differ significantly,

the ratio between pyruvate and acetate incorporation into cholesterol is approximately constant for both hepatomas.

The two sarcomatoid tumours are characterized by a high preponderance of pyruvate incorporation over that of acetate into cholesterol.

DISCUSSION.

The question arises as to what may be the regulatory mechanisms which govern the direction of flow of the two-carbon fragments to the diverse metabolic products in the neoplastic tissues and to what extent these mechanisms may be characteristic for a group of tumours.

Acetate seems to be superior to pyruvate as a carbon source for the formation of higher fatty acids in liver slices (Bloch, 1952 ; Anker, 1948). This is primarily because pyruvate, in contrast to acetate, is converted for the greater part to glycogen and lactic acid. Except for the hepatomas, practically no glycogen storage occurs in the other tumours and hence the competing enzymes, distracting pyruvate from this alternative metabolic pathway, are not engaged. The rates to which acetate-1-C^{14} or pyruvate-2-C^{14}, which both yield 1-C^{14}-acetyl-coenzyme A, are incorporated into the substances isolated in this investigation depends among other things on the relative *effective* concentrations of competing enzymes and coenzymes. The potential concentration of coenzyme A (CoA) was determined in five of the six tumours (Emmelot and Bosch, 1955*a*, 1955*b*). The results showed that the content of this enzyme paralleled roughly the synthetic abilities of the tumours, although it was demonstrated that, in general, the level of CoA need not be the rate limiting factor in biosynthesis.

Preliminary experiments seem to indicate that the alternative reaction : pyruvate $\rightarrow$ lactate may play some role, since a greater amount of lactate is produced by the hepatomas (1·0–1·2 mg. lactic acid per g. wet weight of tumour slices from 3 mg. pyruvate in 1 hour) under aerobic conditions than by the four remaining tumours (0·7–0·8 mg. lactic acid).

Another important feature is the energy supply necessary for the initial activation reaction of acetate to acetyl-CoA, in contrast with the formation of acetyl-CoA starting from pyruvate which needs no adenosine triphosphate. This may have its bearing on the metabolic processes in the different tumours. There is ample evidence from this and the preceding investigation, that the sarcomatoid ovarian tumours oxidize acetic acid at a lower rate than the other tumours studied, whereas a pronounced preference of the sarcomatoid tumours for pyruvate utilization in all the metabolic reactions was noted.

At the moment there seems no simple answer to the question why the tumours display such quantitative differences in metabolic behaviour, as expressed by the preferential utilization of acetate or pyruvate for the synthesis of certain cell constituents. In Table IV the ratio between the incorporation of tracer from pyruvate and acetate for the various compounds, isolated from the six tumours, are given. In considering the data from this table, the three tumour groups may be listed with increasing ability to utilize pyruvate relative to acetate in the order, hepatomas, ovarian tumours of the granulosa cell type, and ovarian tumours of the sarcomatoid type.

Despite individual variations in the ratios of C^{14} incorporation and the limited number of tumours studied, it may appear from this that regulatory mechanisms

TABLE IV.—*Ratio between the Incorporation of C^{14} from Pyruvate-2-C^{14} and Acetate-1-C^{14} into CO_2, Protein, Cholesterol and Fatty Acids.*

Tumour.	CO_2.	Proteins.	Cholesterol.	Fatty acids.
T24202	6·8	9·9	21	5·0
	6·4	9·8	9·9	—
	25	20	26	2·7
T26567	5·5	7·3	3·6	3·5
	9·8	13·3	8·0	5·7
T19957	4·6	14·5	1·3	1·4
	3·8	5·0	1·2	9·7
	5·2	7·1	1·4	4·7
T 5441	1·8	2·3	0·7	1·7
	1·8	7·4	1·8	3·3
	6·4	8·6	2·6	2·6
T26473	2·4	3·5	0·75	0·21
	2·9	2·9	0·97	0·30
	4·2	4·0	1·5	1·2
Spontaneous hepatoma	1·8	1·6	0·92	0·95
	1·4	2·7	0·90	0·97

are operative which control the rate of flow of the carbon intermediates derived from pyruvate, with respect to those derived from acetate, in a relatively similar manner in those tumours which belong to the same group.

It must be emphasized that the tumours chosen in the present investigation—two in each group—differ significantly in both their origin and growth rate. In the group of hepatomas, spontaneous and transplanted tumours were studied. The transplanted hepatomas usually took 2–2·5 months per generation, Tumour 5441 of the granulosa cell type 1 month and Tumour 19957 4–5 months. The two sarcomatoid types, on the other hand have a fast growth rate (3 weeks). Hence the biochemical features found are not simply due to the rate of tumour growth *per se*.

The scope of this work does not permit us to state whether similar biochemical characteristics can be ascribed to other tumours but it is hoped to study this as soon as possible.

SUMMARY.

1. The relative rates of pyruvate and acetate incorporation into carbon dioxide, protein-bound amino acids, cholesterol and higher fatty acids were studied in three different groups consisting of two tumours each.

2. The ratio between pyruvate and acetate incorporation appeared to furnish a fair means of evaluating differences in metabolic behaviour and of classifying the six tumours studied into three groups.

3. The tumours could be listed with an increasing ability to utilize pyruvate relative to acetate in the order: hepatomas, ovarian tumours of the granulosa cell type, and ovarian tumours of the sarcomatoid type.

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SURVEY OF PAPERS

VERSLUYS compares the mammary cancer mortality in the various provinces of the Netherlands and finds that for married women an inverse correlation exists between marriage fertility and mammary carcinoma mortality. This is shown by a low mammary cancer mortality in provinces with a high marriage fertility and *vice versa* ; no such variation from province to province is seen in the mortality of single women from this condition (p. 239).

MOORE and BARR report a study of the sex characteristics of nuclei from a series of human benign tumours and related conditions. They find well developed masses of sex chromatin in the nuclei of specimens from females only, as in normal tissues (p. 246).

GUIMARAES, LAMERTON and CHRISTENSEN review the literature on the late effects of thorotrast, with particular reference to its carcinogenic action. They also report and discuss the effects of intravenously administered thorotrast in mice, with and without additional whole body irradiation (p. 253).

BERENBLUM and HARAN report that papillomas developed in half the mice treated with a single skin application of dimethylbenzanthracene followed by 40 twice-weekly applications of croton oil, whereas only one mouse out of 20 developed tumours when the croton oil applications preceded the application of the dimethylbenzanthracene (p. 268).

GILLMAN, PENN, BRONKS and ROUX have studied the process of healing in human skin injuries of various types, including the donor sites of Thiersch-grafts. They conclude that epidermal invasions of the dermis are normal concomitants of the repair of acute and chronic skin injuries, and that the supervention of such invasion is related to the activity of the connective tissue. Disturbances of the connective tissue in healing wounds may alone bring about markedly increased hyperplasia and invasiveness of the epithelium (p. 272).

MACKENZIE describes the production of malignant mammary tumours in rats of three different strains by the implantation of oestradiol, oestradiol dipropionate or stilboestrol (p. 284).

FIRTH and ROE find no support for the suggestion that an infective agent is concerned in the production of the lung adenomata which develop in mice after urethane treatment (p. 300).

COOPER and LINDSEY have detected 3 : 4-benzpyrene in addition to a number of other polycyclic hydrocarbons in tobacco smoke obtained by smoking cigarettes in a machine designed to simulate normal human smoking. The amount of benzpyrene in the smoke from 500 cigarettes was 4·0 μg. (p. 304).

CRABTREE reports the effect of incorporating azo-compounds, potential precursors of p-aminobenzoic acid, in the diet of rats being given the carcinogen 4-dimethyl-aminoazobenzene. All five azo-compounds tested showed a common, but variable inhibitory action on liver-tumour induction (p. 310).

CHALMERS finds that benzpyrene added *in vitro* to human or animal serum is adsorbed by the lipoprotein fraction. The blood serum of rats and fowls contains both unchanged and metabolised benzpyrene after intravenous injection of the hydrocarbon ; the unchanged benzpyrene is found to be associated with the globulin fraction of the serum, the metabolite with the albumin. The nature of these associations is discussed (p. 320).

EMMELOT and BOSCH have investigated the rate of synthesis of cholesterol and fatty acids by oestrogenic ovarian tumours and hepatomas in mice both *in vitro* and *in vivo*. The process of biosynthesis was studied using C^{14} labelled sodium acetate as a substrate (p. 327).

EMMELOT and BOSCH have studied the incorporation of radioactive carbon labelled acetate into carbon dioxide, protein, cholesterol and fatty acids by slices of mouse tumour transplants with and without fructose and glucose in the medium. They find that fatty acid synthesis is markedly stimulated by glucose, but less so by fructose; cholesterol synthesis is also stimulated, but to a lesser degree. They conclude that the enhancement of fatty acid synthesis is directly related to the active glycolysis of the tumour tissues (p. 339).

EMMELOT and BOSCH have studied the relative rates of pyruvate and acetate incorporation into carbon dioxide, protein-bound amino acids, cholesterol and higher fatty acids in several mouse tumours *in vitro*. They find that the tumours investigated can be listed with an increasing ability to utilise pyruvate relative to acetate in the order: hepatomas, granulosa cell ovarian tumours and sarcomatoid types of the latter (p. 344).